REMARKS

Claims 1-17, 25 and 26 are withdrawn in view of Applicant's election in response to the Restriction Requirement mailed on June 23, 2003.

Claims 27-44 are newly added herein. Support for the newly added claims is found, for example, in the original specification on page 5, lines 3-10, and on page 8 the paragraph bridging pages 8 and 9. Hence no new matter is presented.

Applicant thanks the Examiner for the courtesies extended during the Interview conducted on June 16, 2004. A Statement of the Substance of the Interview was previously submitted on July 16, 2004, in accordance with 37 C.F.R. § 1.133.

An RCE, Petition for Extension of Time and a Declaration under 37 C.F.R. § 1.132 are submitted herewith.

I. Response to Claim Rejections under 35 U.S.C. § 112, 1st Paragraph

Claims 20-24 are rejected under the first paragraph of 35 U.S.C. § 112, as being non-enabled by the specification. Specifically, it is the Examiner's position that the specification does not teach one of ordinary skill in the art how to make and use the invention commensurate in scope with the claims because there is a lack of teaching in the specification as to which compounds are derivatives of serine or alanine; what quantities of the components are required; which specific compounds are hydrophobic derivatives; and what compounds have OH attached to C or N.

Applicant respectfully traverses the rejection. Specifically, Applicant disagrees with the Examiner's position that undue experimentation is required to practice the invention. As

previously stated in the response filed on December 23, 2003, the compounds listed in the Appendix attached to the Amendment were discovered using the *in vitro* and *in vivo* assays that were disclosed in the present application. Construction of compound libraries based upon functional assays is a standard chemical approach. Since submission of the original application, using the concept of claims 22 and 23, Applicant has obtained a library consisting of over 200 compounds meeting the description of the compounds in the specification (i.e., hydrophobic group attached to either the C or N of D-serine), as illustrated in the Appendix attached the Amendment and filed on December 23, 2003. Several of these compounds have shown the ability to inhibit D-serine transport using the assay system described herein. Generation of these compounds was performed by one of ordinary skill in the art, and testing was performed according to the specifications provided. Thus, the specification has proven to be enabling to individuals skilled in the art, contrary to assertions of the Examiner.

As detailed in *In re Wands* the test regarding undue experimentation is not merely quantitative since "a considerable amount of experimentation is permitted to practice an invention if it is merely routine or if the specification in question provides a reasonable amount of guidance." In the attached Declaration under 37 C.F.R. § 1.132 that all experimentation performed to identify active compounds was performed using procedures disclosed in the submitted application, and, as detailed, these experiments yielded compounds with behavioral characteristics as reflected in the original application. Thus, as per *In re Wands*, no undue experimentation was required.

In summary, since the skilled artisan can readily determine derivatives of serine or alanine usable in the present invention based upon the guidance provided and knowledge within the art, Applicant's claims 20-24 are clearly enabled by the application as filed.

Applicant further submits that the present specification provides sufficient enablement for the present claims consistent with and similar to other issued U.S. patents. For example, Applicant refers to U.S. Patent No. 6,150,349 (copy attached), with claims to a method of ameliorating psychosis in a patient in need thereof by administering an effective amount of glucocorticoid receptor antagonist. See claim 1. Claim 2 of the '349 Patent recites that the glucocorticoid receptor antagonist comprises a steroidal skeleton with at least one phenyl-containing moiety at the 11 beta position of the steroidal skeleton. Further, a glucocorticoid receptor antagonist is defined in the description as "any composition or compound which partially or completely inhibits (antagonizes) the binding of a glucocorticoid receptor (GR) agonist". See column 4, line 64 to column 5, line 1. A glucocorticoid receptor antagonist is also defined as "any composition or compound which inhibits any biological response associated with the binding of a GR to an agonist." Col. 5, lines 1-4.

Based upon the presumption of validity under 35 U.S.C. § 282 of these claims of the '349 Patent, Applicant submits that the present specification is at least equally enabling for the claimed D-serine transport inhibitors based upon the disclosure of a D-serine or D-alanine skeleton and the description in the present specification for identifying d-serine transport inhibitors within the scope of the claims. Thus, Applicant submits that, clearly, the need for synthesis and screening compounds belonging to a specified genus, even if that screening

requires trial and error, has not been considered undue experimentation under *In re* Wands, by precedent.

Accordingly, Applicants respectfully request withdrawal of the rejection under 35 U.S.C. § 112, 1st paragraph, for lack of enablement.

II. Response to Claim Rejections Under 35 U.S.C. § 103

In the Office Action dated April 14, 2004, claims 18-23 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Applicant's admissions. As best as understood, it is believed that the Examiner's position is that D-serine, known in the art, acts as a D-serine transport inhibitor.

Applicant respectfully submits the Examiner's assertion that D-serine and D-serine transport inhibitors speak to the same effect is incorrect. D-serine binds directly to the glycine/D-serine binding site of the NMDA receptor complex to activate NMDA receptors. D-serine transport inhibitors, as described, do not bind to the glycine/D-serine binding site and thus do not substitute directly for D-serine. Instead, they inhibit reuptake of D-serine by D-serine transporters in brain. A novel inventive step of this application was the first demonstration by the inventor of transporters capable of regulating D-serine concentrations *in vivo*.

On page 2, line 12 to page 3, lines 1-2, a clear distinctions is set forth between the concept of "agents that activate the glycine site directly (e.g., glycine, D-serine)" and agents that function "by inhibition of glycine or D-serine transport (e.g., D-ala dodecylamide)." An inventive concept underlying both this and prior applications is that the brain must contain

transporters that regulate endogenous levels of glycine and D-serine even though these had not been described prior to Applicant's research. Applicant's discovery in 1995 of a novel glycine transporter led to invention of the use of glycine transport inhibitors in treatment of schizophrenia (U.S. Patent No. 5,837,730 - Treatment of Negative and Cognitive Symptoms of Schizophrenia with Glycine Uptake Antagonist). At the time those studies were conducted, no D-serine transporters had yet been described. The present application is the first to describe a novel D-serine transport process in the brain. Prior to demonstration of this transporter, development of D-serine transport inhibitors would have been impossible. This invention enables development of D-serine transport inhibitors and their use in schizophrenia.

D-serine does not function as a D-serine transport inhibitor since by definition it is transported by the D-serine transporter. This is obvious based upon Example 1, Figs. 1-3, of the application as filed in which uptake of D-[3H] serine is the primary variable being studied. If D-serine were a D-serine transport inhibitor it would not, by definition, be transported by the D-serine transporter. Further, as demonstrated in these figures, uptake of unlabeled D-serine is able to compete with uptake of D-[3H]serine to reduce total tritium uptake to non-specific levels. The competitive nature of the interaction between tritiated [3H] and non-tritiated forms of D-serine is the hallmark of a competitive transport process. Such transport is not demonstrable for D-serine derivatives that function as D-serine transport inhibitors.

During the Interview conducted on June 16, 2004, the Examiner took the position that the claimed compounds were known previously and that discovery of a new property or use of a previously known composition does not support the patentability of the composition. Further the

Examiner expressed a concern that compounds including D-serine and GDA were previously disclosed. Specifically, the Examiner took the position that the present application was deficient in that it did not demonstrate utility of D-serine transport inhibitors over and above D-serine or GDA.

Applicant respectfully traverses the rejection for the following reasons. Although Applicant agrees that GDA was characterized previously, Applicant disagrees as to the D-serine and D-alanine dodecylamide compounds recited in the claims. These compounds were referred to in Tsai et al only as part of a genus (alkylated derivatives having 1-12 carbons), with no functional assay put forth to select among the many representatives of this group. Thus, the disclosure of Tsai et al is not an enabling disclosure to one of ordinary skill in the art for the recited D-serine and D-alanine dodecylamide compounds and does not destroy the novelty of the presently claimed compounds. Nor does the prior art destroy the novelty of the presently claimed method of using these compounds for inhibition of D-serine transport.

Moreover, the disclosure by Tsai et al does not render the presently claimed D-serine and D-alanine dodecylamide compounds obvious. The presently claimed compounds have a much greater potency relative to D-serine, in producing behavioral effects in rodents. This difference has been reproduced with novel D-serine transport inhibitors in experiments conducted since the original submission (see attached Declaration under 37 C.F.R. § 1.132), confirming the utility of compositions containing D-serine transport inhibitors.

In response to the Examiner's position regarding the utility of the claimed compounds in relation to D-serine, Applicant provides the following detailed distinctions based upon data presented in the original application.

In the application, detailed findings are presented with respect to 3 compounds: GDA, D-Ser-DA, and D-Ala-DA. Effects of these compounds on glycine and D-serine transport are shown in Table 1. The 3 compounds show distinguishable profiles, with GDA producing significant inhibition of both glycine and D-serine uptake at a concentration of 100 – 500 μM, D-Ser-DA showing inhibition only of glycine uptake, and D-Ala-DA showing inhibition only of D-serine uptake. In the original application, data were collapsed across the two compounds that effectively inhibited D-serine transport (GDA, D-Ala-DA) vs. the two that did not (saline, D-Ser-DA) in order to demonstrate the utility of D-serine transport inhibitors. In the present reanalysis, the two drugs with D-serine transport inhibition capability (GDA, D-Ala-DA) are explicitly compared in order to demonstrate the differential utility of pure D-serine transport inhibitor D-Ala-DA vs. the mixed glycine/D-serine transport inhibitor GDA.

As noted in the original application, both compounds which functioned as D-serine transport inhibitors (GDA, D-Ala-DA) significantly enhanced activity following PCP administration, with compounds leading to a similar 30-40% enhancement of activity (Table 2, bottom set of numbers). In contrast, the compounds, taken together, did not significantly inhibit amphetamine-induced hyperactivity, leading to a significant statistical interaction (amphetamine/PCP X serine transport inhibitor). These data support the use of D-serine transport inhibitors, as a class, in treatment of schizophrenia, but were not written with the

intention of distinguishing pure D-serine transporter inhibitors from mixed glycine/D-serine transport inhibitors.

Inspection of Table 2, however, in fact reveals differences between D-Ala-DA (a pure D-serine transport inhibitor) and GDA (a mixed glycine/D-serine transport inhibitor) that predict differential utility in treatment of schizophrenia. Moreover, research performed since that time supports the distinctions apparent in Table 2 as well as clinical data suggesting differential efficacy of the two classes of drug.

In Table 2, D-Ala-DA and GDA do not differ in effect on PCP-induced activity (lines 6 and 8). The two compounds, however, do differ in effects on amphetamine-induced activity (lines 2 and 4). In particular, the mixed glycine/D-serine transport inhibitor GDA did not inhibit amphetamine-induced hyperactivity as reflected in the absence of a statistical difference between GDA (line 2, col 5) and saline (line 1). The pure glycine transport inhibitor D-Ser-DA also did not affect amphetamine-induced hyperactivity (line 3). In contrast, the pure D-serine transport inhibitor D-Ala-DA (line 4) significantly inhibited amphetamine-induced hyperactivity relative to all other compounds ($t=2.41$, $df=30$, $p=.02$) and relative to either GDA ($t=2.21$, $df=14$, $p=.02$) or D-Ser-DA ($t=2.21$, $df=14$, p=specifically . Thus, despite the fact that both D-Ala-DA and GDA have similar effects on D-serine transport, the fact that D-Ala-DA does not additionally inhibit glycine transport whereas GDA does permits D-Ala-DA to inhibit amphetamine induced hyperactivity whereas GDA does not.

An additional point, which was not emphasized in the application but is nonetheless apparent from the presented data, is the differential utility of D-serine transport inhibitors vs. D-serine in terms of dose of medication required. D-serine is conventionally known to inhibit activity induced by NMDA antagonists only at extremely high dose (e.g., 4000 mg/kg ip-Nilsson et al., 1997) (1). In contrast, D-Ala-DA was effective at a dose of 1.6 mg/kg.

Amphetamine-induced hyperactivity in rodents is considered to be a model for positive symptoms of schizophrenia. The ability of a pure D-serine transport inhibitor (D-Ala-DA) to reverse amphetamine-induced hyperactivity relative to a mixed glycine/D-serine transport inhibitor (GDA) or a pure glycine transport inhibitor (D-Ser-DA) thus demonstrates differential utility for this class of compound that could not be predicted based upon prior research. Furthermore, although Tsai et al taught use of D-serine and related derivatives for treatment of schizophrenia, they did not teach differential use of compounds that inhibit D-serine transporters without affecting glycine transporters vs. those that do not.

Studies performed since submission of the original application support the distinction between agents working entirely at the D-serine site and those working at the glycine site or mixed glycine/D-serine. These include the following:

1. Confirmation of the potency of GDA in inhibiting glycine transport (2)
2. Confirmation of the ability of glycine transport inhibitors, such as the recently developed compound NFPS to inhibit PCP-induced hyperactivity, but not amphetamine-induced hyperactivity (3).

In addition, the applicant has continued research on selective D-serine transport inhibitors, as detailed in the attached affidavit. For these studies, a library of over 200 novel compounds were screened for relative potency in inhibiting D-serine vs. glycine transport. Several compounds were studied, of which one compound (ALB204) showed best pharmacological characteristics. This compound showed μM affinity for the D-serine transport inhibitor and was potent in both *in vitro* and *in vivo* models of NMDA activity (see attached report). This compound was therefore evaluated in the same behavioral assay system using to characterize GDA, D-Ser-DA and D-Ala-DA in the original application. A prediction of the studies detailed in the application is that ALB204, which is a selective D-serine transport inhibitor, should show an activity profile similar to that previously reported for D-Ala-DA.

This prediction is, in fact, confirmed by the studies detailed in the attached Declaration under 37 C.F.R. § 1.132. For these studies, an initial set of PCP studies was performed using C57 mice as described in the application. Effects of both D-serine (2525 mg/kg) and ALB204 (50 mg/kg) were measured. As predicted, both agents significantly stimulated locomotor activity following PCP administration. However, the degree of stimulation was much more robust following ALB204 administration than following D-serine administration despite the substantially lower dose employed. The greater efficacy of the D-serine transport inhibitor vs. D-serine, which was evident as well with D-Ala-DA in the original application, was not anticipated by prior research.

The effectiveness of D-serine and ALB204 on reversing amphetamine-induced hyperactivity was also assessed. For these studies, conditions and measurements were optimized

for demonstration of D-serine effects. In particular, activity was measured using horizontal counts (HC) instead of distance traveled (DT). Further, activity was summed over 0-60 min. Using these parameters, a highly significant effect of D-serine was observed, with activity reduced to <50% of saline control ($t=5.54$, $df=107$, $p<.0001$). With these same parameters, ALB204 produced a similar degree of inhibition ($t=4.64$, $df=81$, $p<.0001$) again despite the markedly lower dose used of ALB204 vs. D-serine. Prior studies using similar parameters have failed to show effects of either glycine or of high affinity glycine transport inhibitors. The present studies thus show differential effectiveness of D-serine transport inhibitors, as a group, compared to either D-serine or mixed glycine/D-serine transport inhibitors such as GDA.

Condition	Baseline		Pretreatment		Amph/PCP challenge	
PCP treatment	DT (cm)	Stddev	DT (cm)	Std dev	DT (cm)	Std dev
Saline	170.65	80.73323	119.7938	103.528	106.2467	90.88883
D-serine	296.5	215.3587	42.8125	33.50499	292.9375	391.0778
ALB204	230.35	113.2418	83.1875	56.71897	459.1714	513.6259
Amphetamine		Std dev	HC	Std dev	HC	Std dev
treatment HC						
Saline	3363.87	1363.011	1736.952	1040.004	9128.286	5316.11
D-serine	3411.47	1349.811	605.2391	342.254	4566.957	1963.639
ALB204	3528.15	1404.695	430.05	333.0438	5143.25	2523.051

In summary, Applicant disagrees with the Examiner's assessment that the disclosure of the '657 application simply provides a mechanism of action for drugs already known to be effective in treatment of schizophrenia, and is anticipated by Tsai et al. In particular, Tsai et al does not teach the existence of a D-serine transport inhibitor, nor does Tsai et al specify that D-serine functions by inhibiting D-serine transport. Further, although Tsai et al refers to both D-serine and D-alanine derivatives having 1-12 carbons, the application does not distinguish between the pharmacological activities of the two sets of compounds, detail the properties by

which appropriate derivatives may be selected, or enable one skilled in the art to develop additional compounds with desired chemical functionality. Based upon the Tsai et al disclosure, it would not have been obvious to one skilled in the art that compounds having structures other than those described in the application but nonetheless having capacity to inhibit D-serine transport would nonetheless be effective in treatment of schizophrenia.

In contrast, the demonstration of the differential characteristics of GDA, D-Ser-DA, and D-Ala-DA in the original application, along with the novel D-serine transport system, would permit prediction of differential clinical utility of compounds showing capacity to selectively block the D-serine transporter vs. those lacking this capacity. As demonstrated in the attached Declaration under 37 C.F.R. § 1.132, moreover, the methods provided in the present application as originally filed permitted the applicant to develop further novel compounds with the predicted behavioral characteristics. We respectfully submit that our ability to develop novel compounds based upon methods put forth in the application demonstrates that the disclosure is enabling for one of ordinary skill in the art.

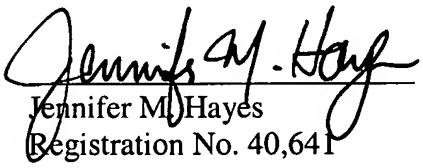
In view of the above, Applicants respectfully submit that the presently claimed compounds are novel and unobvious over the prior art and also provide unexpectedly superior results when compared to the closet prior art examples. Accordingly, Applicants respectfully request withdrawal of the rejection under 35 U.S.C. § 103.

In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the

Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,



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RAPID COMMUNICATION

Daniel C. Javitt · Mark Frusciante

Glycyldodecylamide, a phencyclidine behavioral antagonist, blocks cortical glycine uptake: implications for schizophrenia and substance abuse

Received: 17 June 1996 / Final version: 16 September 1996

Abstract *N*-Methyl-D-aspartate (NMDA) antagonists induce psychotomimetic effects in humans that closely resemble negative and cognitive symptoms of schizophrenia. NMDA agonists, in contrast, may significantly ameliorate such symptoms. In rodents, phencyclidine (PCP) and other NMDA antagonists induce a hyperlocomotory syndrome that is reversed by NMDA agonists. The present study investigates the mechanism of action of glycyldodecylamide (GDA), a drug that is 80-fold more potent than glycine in reversing PCP-induced hyperactivity in rodents. At concentrations relevant to its behavioral actions, GDA significantly inhibits forebrain glycine uptake, indicating that glycine uptake inhibition may provide effective treatment for PCP psychosis and PCP psychosis-like symptoms of schizophrenia.

Key words Phencyclidine · *N*-methyl-D-aspartate receptors · Glutamate · Glycine · Schizophrenia · Drug abuse

Phencyclidine (PCP) induces a psychotic state that closely resembles schizophrenia by blocking neurotransmission mediated at *N*-methyl-D-aspartate (NMDA)-type glutamate (GLU) receptors, indicating that endogenous NMDA receptor dysfunction or dysregulation may play a crucial role in the pathophysiology of schizophrenia (Javitt and Zukin 1991). PCP-like agents uniquely reproduce negative and cognitive symptoms of schizophrenia and uniquely rekindle presenting symptoms in remitted schizophrenic subjects, indicating that the PCP/NMDA model of schizophrenia may be particularly applicable to the pathophysiology of negative and cognitive symptoms of schizophrenia.

The validity of the PCP/NMDA model has been supported recently by the demonstration that NMDA potentiating agents, including glycine (GLY) (Javitt et al. 1994; Heresco-Levy and Javitt 1996; Leiderman et al. 1996) and D-cycloserine (Goff et al. 1995), significantly ameliorate negative symptoms in neuroleptic-treated patients. This finding also indicates that behavioral tests that are sensitive to NMDA-mediated GLY effects may be particularly valuable in drug development. In rodents, PCP induces transient hyperactivity (Toth and Lajtha 1986) that is observed even in monoamine-depleted animals (Carlsson and Carlsson 1989). GLY reverses PCP-induced hyperactivity in doses similar to those which are effective clinically. Other GLY agonists, such as D-serine or D-alanine (administered icv), also reverse PCP-induced hyperactivity (Tanii et al. 1994), indicating that this assay may be effective for identifying behaviorally effective NMDA augmenting agents. To date, two drugs have been identified that are more potent than GLY in reversing PCP-induced hyperactivity following peripheral administration (Toth et al. 1986). The first of these, glycineamide (GCA), acts as a GLY prodrug and elevates CNS GLY levels following peripheral administration. Thus, its behavioral effectiveness is relatively easily understood. However, the second of these agents, glycyldodecylamide (GDA), does not increase CNS GLY levels even though it is approximately 80-fold more potent than GLY itself in antagonizing PCP-induced hyperactivity. GLY levels in brain are regulated by the activity of several amino acid transporters, including the high-affinity, low capacity áglyá system (Barker and Ellory 1990) which corresponds to the actions of the GLYT1 glycine transporter (Guastella et al. 1992). Isoforms of GLYT1 are colocalized with NMDA receptors in brain and may maintain low GLY levels specifically in the local region surrounding NMDA receptors (Smith et al. 1992). Blockade of GLYT1 would thus be expected to elevate GLY levels at NMDA receptors without of necessity altering whole brain levels which may be regulated by transporters other than GLYT1. The present study investigates the hypothesis that GDA might mediate its potent anti-PCP effects by blocking GLYT1-mediated glycine uptake in cortex, thereby augmenting NMDA receptor-mediated neurotransmission.

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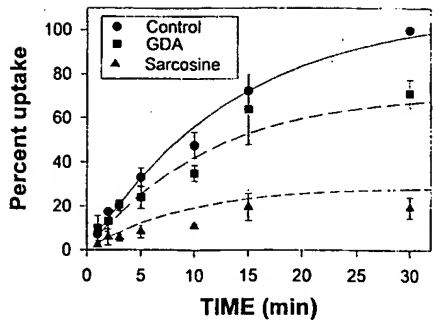

Fig. 1 Kinetics of [^{3}H]glycine uptake into P_2 synaptosomes. Points represent means ± SEM of three separate experiments, each performed in triplicate

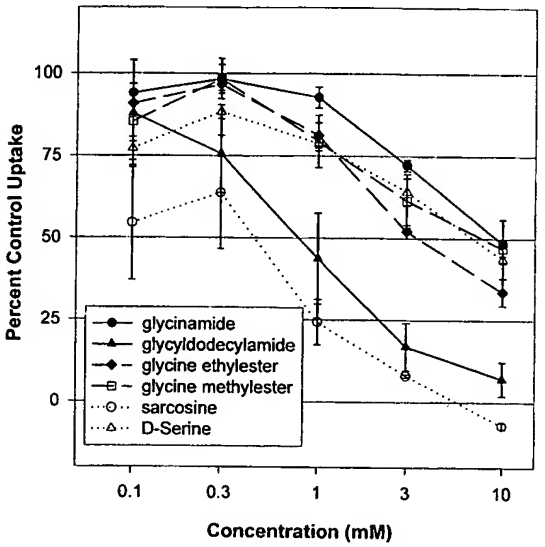

Fig. 2 Inhibition of [^{3}H]glycine uptake by indicated glycine derivatives. Points represent means ± SEM of three separate experiments, each performed in triplicate

For GLY uptake studies, synaptosomal P_2 fractions were prepared from cortex of adult Sprague-Dawley rats and suspended in artificial CSF. Uptake of 100 nM [^{3}H]GLY was measured at 25°C for the indicated time period in the presence of indicated ligands. Incubation was terminated by filtration under reduced pressure. Uptake was linear for at least the first 10 min of incubation (Fig. 1). Apparent plateau was reached at 30 min, with no significant increase in uptake between 30 and 60 min. Kinetic binding parameters were determined by nonlinear regression. All uptake curves were determined to be first order. The mean $t_{1/2}$ value was 9.7 ± 0.7 min. The IC_{50} value for inhibition of [^{3}H]GLY uptake by cold GLY was 78.7 ± 37.8 μM. Specific [^{3}H]GLY uptake was abolished in the absence of added $Na^+/Cl^á$.

Inhibition studies were conducted following 5 min incubation in the absence and presence of GDA concentrations between 0.1 and 10 mM (Fig. 2). Several comparison agents were also tested, including (1) sarcosine, a known high potency GLY uptake antagonist, and (2) glycine ethyl ester (GEE) and glycine methyl ester (GME), agents with known lower affinity for the cortical GLY uptake site (Smith et al. 1992). The rank order of potency for inhibition of GLY uptake was GLY > sarcosine > GDA > GEE > GME. Both GCA and D-serine showed IC_{50} values of >10 mM. Effects of GDA and sarcosine remained significant throughout the 30-min incubation period. Concentrations of 1 mM GDA and sarcosine significantly ($P<0.05$) reduced the maximal level of [^{3}H]GLY uptake by 29 ± 7 and 72 ± 3%, respectively (Fig. 1). GDA 5 mM decreased maximal [^{3}H]GLY uptake by 51 ± 13.3. Effects of these agents on the rate constant of GLY uptake were not significant, although $t_{½}$ values in the presence of both GDA (7.9 ± 2.9 min^{á1}) and sarcosine (6.3 ± 1.9 min^{á1}) were somewhat lower than under baseline conditions. Given that the effective concentration of GDA in behavioral studies is approximately 0.3 mmol/kg, these studies demonstrate that GDA acts as a GLY uptake antagonist at a concentration similar to what may be obtained in behavioral studies.

Potential direct agonist-like effects of GDA at the GLY site of the NMDA receptor complex were excluded using PCP receptor binding as a functional probe of NMDA receptor activation. In this assay, GLY-like agents stimulate [^{3}H]MK-801 binding in the presence,

Table 1 Effect of glycine and glycine derivatives on [^{3}H]MK-801 binding.Data represent means of four experiments, each performed in triplicate. Repeated measures ANOVA revealed significant across-condition variation in binding ($F_{3,9} = 14.6$, $P<0.0001$). Post-hoc analyses were performed using Student-Newman-Keuls Test. * $P<0.05$ vs control and glutamate alone. ** $P<0.05$ vs glutamate + glycine

Agent	Specific binding (DPM)
Control	33 ± 14
Glutamate (10 μ M) alone	296 ± 58
Glutamate (10 μ M) + glycine (10 μ M)	720 ± 145*
Glutamate (10 μ M) + glycyldodecylamide (1 mM)	337 ± 48**

Table 2 Effects of glycine and glycine derivatives on [^{3}H]GABA and L-[^{3}H]glutamate uptake. Data represent means of three experiments, each performed in triplicate. All agents were tested at 1 mM concentrations. * Significantly greater than control binding $P<0.05$. ** Significantly less than control binding, $P<0.05$. *** Significantly less than control, $P<0.0001$. *n.d.* not determined

Agent	Percent control L-[^{3}H]glutamate uptake	Percent control [^{3}H]GABA uptake
Glycine (GLY)	105 ± 13	86 ± 2**
Glycineamide (GCA)	122 ± 18	112 ± 5
Glycyldodecylamide (GDA)	153 ± 17*	180 ± 17*
Nipecotic acid	n.d.	4 ± 0.4***
L-*Trans*-pyrollidine-2,4-dicarboxyllic acid (L-PDC)	9 ± 2***	n.d.

but not absence, of NMDA agonist (GLU). Assays were performed using crude synaptic membranes prepared from rat cortex and hippocampus and incubated for 15 min in 5 mM TRIS-acetate buffer (pH 7.4) in the presence of 1 nM [^{3}H]MK-801 and indicated ligands. Incubation was terminated by filtration under reduced pressure through Whatman GF/B filters. Nonspecific binding was determined in the presence of 10 μM MK-801. Incubation with GLU alone led to a 9-fold increase in binding compared to control conditions (Table 1). Incubation with GLU and GLY led to a highly significant 22-fold increase compared to control conditions and a significant 2.5-fold increase compared to GLU alone. Binding in the presence of combined GLU and GDA was comparable to binding in the presence of GLU alone.

Specificity of GLY uptake antagonism by GDA was examined by evaluating the effects of GLY, GCA and GDA on uptake of GABA and GLU. Methods were the same as in the GLY uptake assay, except that 10 nM concentrations of [^{3}H]GABA or L-[^{3}H]GLU were substituted for [^{3}H]GLY. L-*Trans*-pyrollidine-2,4-dicarboxyllic acid (L-PDC) and nipecotic acid were used as active controls for the GLU and GABA uptake assays, respectively. As opposed to its effects on [^{3}H]GLY uptake, GDA significantly increased uptake of [^{3}H]GLU and [^{3}H]GABA (Table 2). Given that activation of the GLY transporter in brain leads to presynaptic GLU (Bonanno et al. 1994) and GABA (Raiteri et al. 1992) release, potentiation of GLU and GABA uptake is an expected in vitro consequence of glycine reuptake inhibition.

Studies of [^{3}H]GLY uptake into cortical P_2 fraction have consistently demonstrated high (μM) and low (mM) affinity components. The present study, which utilized a submicromolar [^{3}H]GLY concentration, shows pharmacological characteristics of GLYT1-mediated GLY transport. As opposed to GLYT2 transporters, which are confined primarily to hindbrain, GLYT1 transporters are expressed throughout the brain. In hippocampus, moreover, the pattern of GLYT1 expression coincides with that of NMDA receptors, indicating that a primary role for them may be to maintain low GLY concentrations in the immediate NMDA receptor vicinity (Smith et al. 1992).

In summary, this study demonstrates that, among agents which have been shown to reverse the behavioral effects of PCP in vivo, GDA has a unique mechanism of action. Other NMDA modulatory agents that reverse PCP-induced hyperactivity function either as direct agonists (e.g., GLY, D-serine) or as GLY prodrugs (e.g., GCA). GDA does not increase GLY levels in vivo, and therefore does not act as a GLY prodrug (Toth et al. 1986), and does not stimulate PCP receptor binding (this study), indicating that it does not act as a direct GLY agonist. However, GDA does inhibit cortical GLY uptake at doses similar to those that are behaviorally effective. Given the potent behavioral activity of GDA, this finding indicates that agents which inhibit GLY uptake may be as, or more, effective than direct GLY agonists in the treatment of schizophrenia.

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The Role of Glycine$_B$ Binding Site and Glycine Transporter (GlyT1) in the Regulation of [^{3}H]GABA and [^{3}H]Glycine Release in the Rat Brain*

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The effect of N-methyl-D-aspartic acid (NMDA), a selective glutamate receptor agonist, on the release of previously incorporated [^{3}H]γ-aminobutyric acid(GABA) was examined in superfused striatal slices of the rat. NMDA (0.01 to 1.0 mM) increased [^{3}H]GABA overflow with an EC_{50} value of 0.09 mM. The [^{3}H]GABA releasing effect of NMDA was an external Ca^{2+}-dependent process and the GABA uptake inhibitor nipecotic acid (0.1 mM) potentiated this effect. These findings support the view that NMDA evokes GABA release from vesicular pool in striatal GABAergic neurons. Addition of glycine (1 mM), a cotransmitter for NMDA receptor, did not influence the NMDA-induced [^{3}H]GABA overflow. Kynurenic acid (1 mM), an antagonist of glycine$_B$ site, decreased the [^{3}H]GABA-releasing effect of NMDA and this reduction was suspended by addition of 1 mM glycine. Neither glycine nor kynurenic acid exerted effects on resting [^{3}H]GABA outflow. These data suggest that glycine$_B$ binding site at NMDA receptor may be saturated by glycine released from neighboring cells. Glycyldodecylamide (GDA) and N-dodecylsarcosine, inhibitors of glycineT1 transporter, inhibited the uptake of [^{3}H]glycine (IC_{50} 33 and 16 μM) in synaptosomes prepared from rat hippocampus. When hippocampal slices were loaded with [^{3}H]glycine, resting efflux was detected whereas electrical stimulation failed to evoke [^{3}H]glycine overflow. Neither GDA (0.1 mM) nor N-dodecylsarcosine (0.3 mM) influenced [^{3}H]glycine efflux. Using Krebs-bicarbonate buffer with reduced Na^+ for superfusion of hippocampal slices produced an increased [^{3}H]glycine outflow and electrical stimulation further enhanced this release. These experiments speak for glial and neuronal [^{3}H]glycine release in hippocampus with a dominant role of the former one. GDA, however, did not influence resting or stimulated [^{3}H]glycine efflux even when buffer with low Na^+ concentration was applied.

KEY WORDS: Glycine$_B$ binding site; glycineT1 transporter; [^{3}H]GABA release; [^{3}H]glycine release; striatal slices; hippocampal slices.

INTRODUCTION

The action of glycine in the central nervous system is mediated through two sites: the strychnine-insensitive site (glycine$_B$ site) on N-methyl-D-aspartate (NMDA) receptor and the strychnine-sensitive inhibitory glycine receptor (glycine$_A$ site) located in the spinal cord (1). Glycine and glutamate act as cotransmitters for opening the NMDA-sensitive ionotropic glutamate receptors (2). In 1987 Johnson and Ascher demonstrated that glycine amplifies the agonist action of NMDA at its respective receptor (3). Besides glutamate and glycine, which bind to distinct sites on NMDA receptor, several other compounds also bind as allosteric modulators influencing membrane permeability for mono- and bivalent cations (4). The function

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of NMDA receptor-ion channel complex can be modulated by drugs acting as agonists on glycine recognition site, a number of D-amino acids (D-serine, D-cycloserine, D-alanine) may act as agonist at glycine$_B$ site (5). Kynurenic acid and a number of other compounds (L-683,334, MDL-105 519, CGP 68730A) act as selective antagonist at glycine coagonist site of NMDA receptors by displacing bound endogenous glycine (6).

Although activation of NMDA receptors requires occupancy of glycine binding sites it is a question of debate whether these binding sites are normally saturated at excitatory synapses (7). Several lines of evidence indicate that a potent glycine transport mechanism assures to set glycine concentration below the level required to saturate the glycine site of NMDA receptors (8). In addition, drugs inhibiting glycine uptake enhance endogenous glycine levels in the brain as was shown by microdialysis studies (9,10). The high affinity glycine transport system which primarily inactivates synaptic glycine in the central nervous system, is situated in membranes of neurons and glial cells (11).

Glycine transporters are members of the Na^+- and Cl^--dependent neurotransmitter transporter family (12,13). Two glycine transporter genes (GlyT1 and GlyT2) have been identified and cloned (14,15). In addition, three isoforms for glycine T1 (GlyT1a, GlyT1b and GlyT1c) were identified and two isoforms for glycineT2 (GlyT2a and GlyT2b) have been isolated, cloned and characterized (16,17). Glycine transporters which regulate glycine concentrations in excitatory synapses belong to glycine T1 transporters and the transport proteins have been shown to be colocalized with NMDA receptors (18,19).

A number of compounds have been reported to have selective and high affinity to glycine transporters. Toth and Lajtha (20) and Toth and his coworkers (21) have reported that glycine inhibited PCP-induced hyperactivity in mice and glycyldodecylamide (GDA) was found particularly active in this respect. Later, the glycine reuptake inhibitory effect of GDA has been demonstrated (22,23). It has been reported that ORG 24598 (10) and ALX-5407 (24) are potent inhibitors of glycineT1 transporter with little affinity to glycineT2 transporter. The aim of the present investigation was to further characterize the role of glycine$_B$ binding site in the regulation of NMDA-mediated [^{3}H]GABA release. The contribution of glycineT1 transporter in the regulation of [^{3}H]glycine release was also studied in rat brain slices. We found that NMDA receptors which induce vesicular GABA release, possess glycine$_B$ binding sites saturated by endogenous glycine and the source of glycine release may be the neighboring neurons and glial cells.

EXPERIMENTAL PROCEDURE

Preparation of Synaptosomal P_2 Fractions. Synaptosomal P_2 fraction was prepared as described by Gay and Whittaker (25). Male rats weighing 200–250 g were decapitated, the brain was removed from the skull and the hippocampi were dissected and placed into ice-cold saline. The tissue was homogenized in 40 volumes (w/v) of 0.32 M sucrose. The homogenate was centrifuged at 1,000 × g for 5 min to remove nuclei and debris then the supernatant was centrifuged again at 12,000 × g for 20 min. The P_2 pellet was resuspended in Krebs solution with the following composition in mM: NaCl 125, KCl 3, $CaCl_2$ 1.2, NaH_2PO_4 1, $MgSO_4$ 1.2, $NaHCO_3$ 22, glucose 10, pH 7.4 and gassed with 95% O_2/5% CO_2.

Measurement of [^{3}H]Glycine Uptake Inhibition. [^{3}H]Glycine uptake in synaptosomal P_2 fraction prepared from rat hippocampus was carried out as described by Fedele and Foster (26). Aliquots of the P_2 fraction (0.25 mg protein) were preincubated in 2 ml of Krebs solution for 5 min at 37°C. [^{3}H]glycine was then added and the incubation continued for 3 min. The assay mixture was filtered under vacuum through Whatman GF/B filters which were rapidly washed with 3 × 5 ml of Krebs solution at room temperature. Blanks were determined by incubation at 0°C. Filters were placed into scintillation vials with 3 ml of scintillation liquid and radioactivity was measured by liquid scintillation spectrometry. To achieve the desired ligand concentrations, [^{3}H]glycine was diluted by addition of unlabeled glycine. In the experiments, 0.03 mM [^{3}H]glycine was used and the drugs tested were present from the beginning of the experiments at concentrations of 10 to 100 μM.

Preparation of Brain Slices. Male Sprague-Dawley rats weighing 200–250 g were killed by decapitation and the brain was removed from the skull. Coronal slices approximately 350 μm thick were cut from the striatum using a McIlwain tissue chopper (The Mickie Laboratory Engineering Co., Gomshall, UK). In other series of experiments, slices from hippocampus were prepared. The slices were collected into ice-cold Krebs-bicarbonate buffer, pH 7.4 with the following composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 1.25, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $NaHCO_3$ 25, glucose 11.5. The Krebs-bicarbonate buffer used throughout the experiments was continuously gassed with 5% CO_2 in O_2.

Measurement of [^{3}H]GABA Efflux. Striatal slices were incubated with [^{3}H]GABA (2.5 μCi/ml) in oxygenated Krebs-bicarbonate buffer for 30 min at 37°C. β-Alanine (1 mM), an inhibitor of GABA uptake in glial cells (27,28) was present in the incubation buffer. The tissues were then transferred into low-volume (300 μl) superfusion chambers (Experimetria Inc., Budapest, Hungary) and superfused with aerated and preheated (37°C) Mg^{2+}-free Krebs-bicarbonate buffer that contained the aminotransferase inhibitor aminooxyacetic acid (0.1 mM). Except where indicated, the GABA uptake inhibitor nipecotic acid was also present in Krebs-bicarbonate buffer in a concentration of 0.1 mM (29). The flow rate was kept at 1 ml/min by a Gilson multichannel peristaltic pump (type M312, Villiers-Le-Bel, France). The superfusate was discarded in the first 60-min period of the experiment then twenty-two 3-min fractions were collected by a Gilson fraction collector (type FC-2038, Middleton, WI, USA). The ionotropic glutamate receptor agonist NMDA was added to the perfusion buffer from fraction 8 for 3 or 6 consecutive 3-min periods.

Measurement of [^{3}H]Glycine Efflux. Slices from hippocampus were incubated with [^{3}H]glycine (2.5 μCi/ml) in Krebs-bicarbonate buffer for 30 min at 36°C. The tissues were then transferred into low volume (300 μl) superfusion chambers and superfused with aerated and preheated (37°C) Krebs-bicarbonate buffer. The flow rate was kept at 1 ml/min by a Gilson multichannel peristaltic pump. The superfusate was discarded for the first 60-min period of the experiments then twenty-two 3-min fractions were collected by a Gilson multichannel fraction collector. The glycineT inhibitor GDA or N-dodecylsarcosine were added to the buffer from fraction 8 and maintained throughout the experiments.

In some experiments the concentration of NaCl in the Krebs-bicarbonate buffer was lowered from 118 to 5 mM while osmolarity was maintained by addition of 113 mM sucrose in order to inhibit glycine transporter proteins. When used, electrical field stimulation of the slices (40 V, 50 Hz frequency, 2 msec impulse duration for 3 min) was carried out by a Grass S88 Electrostimulator (Grass Instruments, Quincy, MA, USA) in fraction 10.

Determination of [^{3}H]GABA and [^{3}H]Glycine Efflux. At the end of superfusion, the tissues were collected from the superfusion chambers and homogenized in 0.8 ml Soluene-100 and an aliquot (100 μl) was processed for determination of tissue content of radioactivity. To determine the radioactivity released from the tissue, a sample (0.8 ml) of the superfusate was mixed with 4.2 ml of liquid scintillation reagent (Ultima Gold, Packard, Groningen, The Netherlands) and subjected to liquid scintillation spectrometry. Previously we have reported that 92–108% of [^{3}H]GABA released from brain slices is in the form of authentic [^{3}H]GABA (30). Furthermore, the metabolic stability of the released [^{3}H]glycine has also been reported (31).

The efflux of [^{3}H]GABA or [^{3}H]glycine was expressed as a fractional rate, i.e., as a percentage of the amount of radioactivity in the tissue at the time the efflux was determined (32). A computer program (Quattro Pro V6.0) was used to estimate the fractional rate of [^{3}H]GABA or [^{3}H]glycine efflux. The effect of a drug on [^{3}H]GABA or [^{3}H]glycine efflux was also expressed by the ratio of fractional release of radioactivity determined in the present (B2) and absent (B1) of drug investigated. The efflux ratio is expressed as B2/B1 throughout this paper. In some experiments the electrical stimulation-induced [^{3}H]glycine overflow was estimated as the mean of the basal [^{3}H]glycine outflow determined before and after stimulation was subtracted from each sample and summed.

Statistical Analysis. One-way analysis of variance (ANOVA) followed by the Dunnett's multiple comparison test, the Student *t*-statistics for two-means and the paired t test were used for statistical analysis of the data. The GraphPad Prism V2.0 software was used to calculate the slopes of concentration-release curves. The mean ± S.E.M. was calculated and the number of independent determinations (n) is indicated. A level of probability (P) less than 5% was considered significant.

Materials. The following drugs were used in this study: N-methyl-D-aspartic acid (NMDA), Tocris-Cookson, Bristol, UK; kynurenic acid, cyclothiazide, Research Biochemicals Inc., Natick, MA, USA; aminooxyacetic acid, β-alanine, (±)nipecotic acid, spermine, Sigma Chemicals Co., St. Louis, MO, USA. Glycyldodecylamide (GDA), N-dodecylsarcosine and GYKI-53 784 (LY303070, 5-(4-aminophenyl)-7-methylcarbamoyl -8(R)-methyl-8,9-dihydro-7H-1,3-dioxolo[4,5-n][2,3]-benzodiazepine) were synthesized by Dr. S. Solyom, Institute for Drug Research Ltd., Budapest, Hungary. γ-[2,3-^{3}H(N)]-aminobutyric acid ([^{3}H]GABA), specific activity 1.5 TBq/mmol and [^{3}H]glycine, specific activity 41.1 Ci/mmol were purchased from New England Nuclear Life Science Products, Boston, MA, USA. All other chemicals used were of analytical grade.

RESULTS

Characterization of NMDA-Induced [^{3}H]GABA Release from Rat Striatal Slices. In the absence of nipecotic acid, the basal [^{3}H]GABA outflow approached a rate of 1.45 ± 0.14 kBq/g in 3 min after a 60-min initial washout period (n = 8). In these experiments, the content of radioactivity in striatal tissue was found to be 649.55 ± 100.32 kBq/g (n = 8). The calculated fractional [^{3}H]GABA efflux at rest was 0.24 ± 0.03% of content released in 3 min. When nipecotic acid was added to the superfusion buffer in a concentration of 0.1 mM, the basal [^{3}H]GABA outflow increased to 3.30 ± 0.25 kBq/g in 3 min which corresponds to a fractional release of 0.74 ± 0.07% of content released in 3 min, the difference was statistically significant ($P < 0.001$, n = 8). Addition of nipecotic acid (0.1 mM) to the superfused tissue did not change the tissue content of radioactivity (407.89 ± 32.37 kBq/g, $P > 0.05$, n = 8).

NMDA added to the superfusion buffer in concentrations of 0.01 to 1 mM for six consecutive 3-min fractions, produced a concentration-dependent increase in [^{3}H]GABA release (Fig. 1). The concentration of NMDA which increased basal fractional [^{3}H]GABA outflow by 50% (EC_{50}) was found to be 0.09 mM. The [^{3}H]GABA efflux declined to prestimulated values as NMDA was withdrawn from the superfusion buffer.

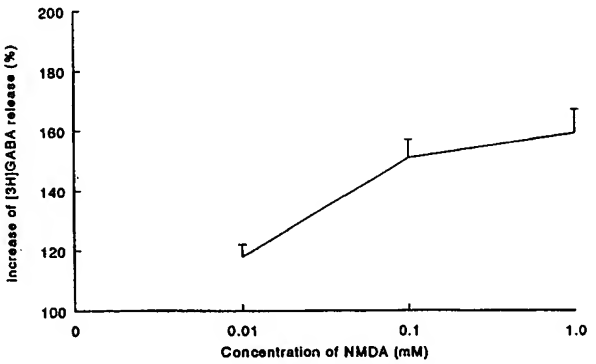

Fig. 1. Effect of NMDA on [^{3}H]GABA release from striatal slices of the rat. The NMDA concentration which increased [^{3}H]GABA overflow by 50% (EC_{50}) was 0.09 mM. Slices from rat striatum were prepared, loaded with [^{3}H]GABA and superfused with Mg^{2+}-free Krebs-bicarbonate buffer containing aminooxyacetic acid and nipecotic acid (0.1 mM). NMDA (0.01 to 1 mM) was added to striatal slices from fractions 5 to 10 (18 min). The fractional release of [^{3}H]GABA was determined in fractions 4 (B1) and 5 (B2) in the present and absent of NMDA and the B2/B1 ratio was calculated. This ratio was 0.92 ± 0.05 in control experiments (n = 4) and it was taken as 100%. Values shown are the mean ± SEM of 3 to 4 experiments.

The effect of 0.3 mM NMDA on [^{3}H]GABA release measured in different experimental conditions in superfused striatal slices is shown in Table I. Addition of nipecotic acid (0.1 mM) to the superfusion buffer increased not only the basal [^{3}H]GABA outflow but also the NMDA-evoked [^{3}H]GABA overflow. In addition, removal of Ca^{2+} from the superfusion buffer in the presence of nipecotic acid significantly decreased the NMDA-evoked [^{3}H]GABA release (Table I).

Cyclothiazide, a positive modulator of AMPA receptor (33), failed to potentiate NMDA-evoked [^{3}H]GABA release: the 0.3 mM NMDA-induced release was 0.74 ± 0.13 in the presence and 0.69 ± 0.07% of content in the absence of 0.03 mM cyclothiazide, respectively ($P > 0.70$ and $n = 3-4$). The negative modulator of AMPA receptors, GYKI-53 784 (0.01 mM) did not reverse the NMDA-induced [^{3}H]GABA release.

Effect of Glycine on NMDA-induced [^{3}H]GABA Efflux. Glycine or spermine was added in a concentration of 1 mM to superfused striatal slices which had been preloaded with [^{3}H]GABA. Neither glycine nor spermine altered the basal [^{3}H]GABA outflow being the B2/B1 ratios 0.99 ± 0.01 in control and 0.99 ± 0.05 and 0.95 ± 0.04 in the presence of glycine and spermine, respectively ($P > 0.70$, $n = 4$). Glycine and spermine in this concentration were also ineffective to modify NMDA-induced [^{3}H]GABA release in striatum (Table II).

Kynurenic acid added in a concentration of 1 mM did not influence basal [^{3}H]GABA outflow in superfused striatal slices of the rat, the B2/B1 ratios were 0.93 ± 0.05 and 0.99 ± 0.03 in the presence and absence of kynurenic acid ($P > 0.60$, $n = 4$). On the other hand, kynurenic acid significantly reduced NMDA-induced release of [^{3}H]GABA in rat striatal slices (Table II). The inhibition by kynurenic acid of NMDA-induced [^{3}H]GABA overflow was reversed by glycine whereas spermine was ineffective (Table II).

Inhibition of [^{3}H]Glycine Uptake. GDA and N-dodecylsarcosine inhibited [^{3}H]glycine uptake with IC_{50} value of 33 and 16 μM, respectively in hippocampal synaptosomal preparations.

Effect of Glycine Uptake Inhibitors on [^{3}H]Glycine Release. After a 60 min preperfusion period, the spontaneous [^{3}H]glycine outflow from hippocampal slices occurred at a rate of 7.92 ± 1.47 kBg/g in 3 min ($n = 4$) and remained essentially at this rate for the following 60 min of superfusion. The content of radioactivity in hippocampal tissue was found to be 284 ± 17 kBq/g ($n = 4$). The calculated fractional [^{3}H]glycine efflux at rest was 2.73 ± 0.20% of content released in 3 min.

The effects of GDA and N-dodecylsarcosine on [^{3}H]glycine outflow were determined in hippocampal slices in a concentration of 0.1 mM which was 3 to 6 times higher than those inhibited [^{3}H]glycine uptake by 50%. Addition of 0.1 mM of GDA to the superfusion buffer did not affect [^{3}H]glycine outflow (Fig. 2.) and N-dodecylsarcosine was found also to be ineffective in applied concentration. Next, hippocampal slices

Table I. Characterization of NMDA-Induced [^{3}H]GABA Release from Rat Striatal Slices

Conditions	Stimulation	[^{3}H]GABA release (B2/B1)	Significance (P)
1. Normal Ca^{2+} Nipecotic acid	NMDA	1.55 ± 0.05	
2. Normal Ca^{2+} Nipecotic acid-free	NMDA	1.28 ± 0.06	1:2 < 0.05
3. Normal Ca^{2+} Nipecotic acid	NMDA	1.51 ± 0.06	
4. Ca^{2+}-free Nipecotic acid	NMDA	1.16 ± 0.10	3:4 < 0.05

Slices from rat striatum were prepared, loaded with [^{3}H]GABA and superfused with Mg^{2+}-free Krebs-bicarbonate buffer containing aminooxyacetic acid (0.1 mM). NMDA (0.3 mM) was added to striatal slices from fractions 8 to 10 (9 min) to evoke [^{3}H]GABA release. Normal concentration of Ca^{2+} in Krebs-bicarbonate buffer was 1.25 mM, when Ca^{2+} was omitted, Na_2EGTA (1 mM) was added to the superfusion buffer. When present, nipecotic acid was added to the superfusion buffer in a concentration of 0.1 mM at the beginning of superfusion. Basal [^{3}H]GABA release in the presence of nipecotic acid (0.1 mM) and normal Ca^{2+} concentration was 1.00 ± 0.02 ($n = 4$). Student *t*-Statistics for two means was used for comparison. Values shown are the mean ± SEM of 4 experiments.

Table II. Effect of Glycine on Kynurenic Acid-Induced Inhibition of NMDA-Evoked [^{3}H]GABA Release from Striatal Slices of the Rat

Compounds	Concentration (mM)	[^{3}H]GABA release (B2/B1)	Significance (P)
1. Control		1.00 ± 0.03	
2. NMDA	0.3	1.41 ± 0.05	1:2 < 0.01
3. NMDA + glycine	0.3 1.0	1.36 ± 0.05	2:3 > 0.05
4. NMDA + spermine	0.3 1.0	1.50 ± 0.08	2:4 > 0.05
5. NMDA + kynurenic acid	0.3 1.0	1.12 ± 0.06	2:5 < 0.05
6. NMDA + kynurenic acid + glycine	0.3 1.0 1.0	1.43 ± 0.05	2:6 > 0.05 5:6 < 0.05
7. NMDA + kynurenic acid + spermine	0.3 1.0 1.0	1.19 ± 0.04	2:7 < 0.05 5:7 > 0.05

Slices from rat striatum were prepared, loaded with [^{3}H]GABA and superfused with Mg^{2+}-free Krebs-bicarbonate buffer containing aminooxyacetic acid and nipecotic acid (0.1 mM). NMDA (0.3 mM) was added to striatal slices from fractions 8 to 10 (9 min) to evoke [^{3}H]GABA release. Kynurenic acid, glycine and spermine were added to the superfusion buffer 30 min before fraction collection started and they were maintained throughout the experiments. ANOVA followed by the Dunnett's multiple comparison test, $F(6,23) = 9.649$, $P < 0.01$. Values shown are the mean ± SEM of 3 to 8 experiments.

loaded with [^{3}H]glycine were stimulated electrically (40 V voltage, 50 Hz frequency, 2-msec impulse duration for 3 min). Electrical stimulation of hippocampal slices with these parameters did not evoke [^{3}H]glycine overflow. Electrical stimulation of the slices was unable to evoke [^{3}H]glycine overflow even in the presence of 0.1 mM of GDA (Fig. 2).

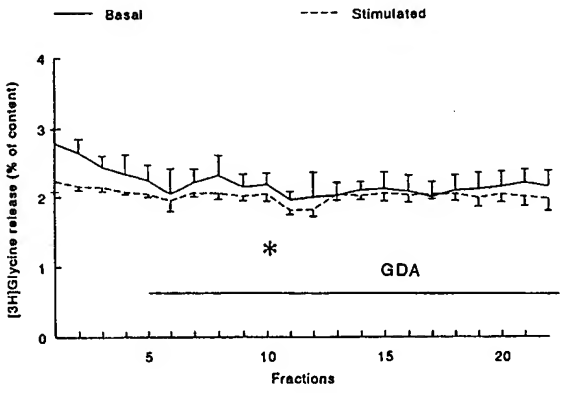

Fig. 2. Glycyldodecylamide (GDA) did not influence resting and electrical stimulation-induced [^{3}H]glycine efflux rate from hippocampal slices of the rat. Slices from rat hippocampus were prepared, loaded with [^{3}H]glycine and superfused with Krebs-bicarbonate buffer. GDA (0.1 mM) was added to hippocampal slices from fraction 5 and maintained throughout the experiments as indicated by the horizontal line. Solid line: effect of GDA on resting [^{3}H]glycine outflow. Broken line: effect of GDA on [^{3}H]glycine efflux when electrical stimulation was applied. * Indicates electrical stimulation (40 V, 50 Hz, 2 msec for 3 min) in fraction 10. Values shown are the mean ± SEM of 4 experiments.

When the superfusion of hippocampal slice preparations was carried out with Krebs-bicarbonate buffer containing Na^+ in reduced concentration (NaCl 5, sucrose 113 mM), the resting [^{3}H]glycine outflow enhanced from 2.17 ± 0.10 to 3.94 ± 0.24% of content released in 3 min ($n = 4$ $P < 0.001$) (Fig. 3). In contrast to hippocampal slices superfused with normal Krebs-bicarbonate buffer, electrical stimulation evoked [^{3}H]glycine release when buffer with reduced Na^+ concentration was used for superfusion (Fig. 3). GDA added to the superfusion buffer in a concentration of 0.1 mM was unable to modify electrical stimulation-induced [^{3}H]glycine overflow even in these conditions: the efflux ratios were 1.26 ± 0.07 and 1.34 ± 0.14 in the presence and absence of GDA ($n = 4$ and 8, $P > 0.70$).

DISCUSSION

In the present study, slices from rat striatum were incubated with [^{3}H]GABA and superfused with Krebs-bicarbonate buffer containing aminooxyacetic acid to minimize the GABA metabolism. NMDA evoked

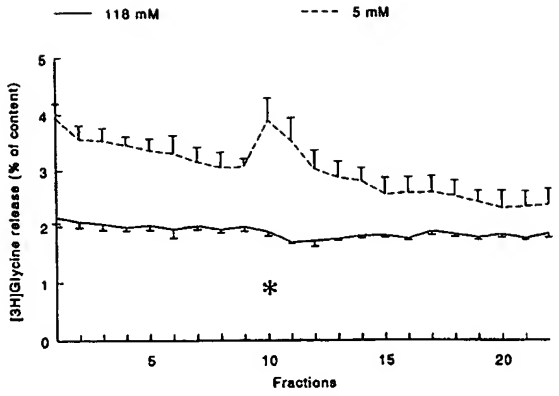

Fig. 3. Electrical stimulation-evoked [^{3}H]glycine efflux rate when Krebs-bicarbonate buffer with reduced Na^+ concentration was used for superfusion. Slices from rat hippocampus were prepared, loaded with [^{3}H]glycine and superfused with two different Krebs-bicarbonate buffers. Solid line: Krebs-bicarbonate buffer with normal composition (NaCl 118 mM). Broken line: Krebs-bicarbonate buffer containing reduced concentration of NaCl (5 mM) and sucrose (113 mM) to maintain osmolarity. The slices were electrically stimulated (40 V, 50 Hz, 2-msec for 3 min) in fraction 10 as * indicates. The electrical stimulation-evoked [^{3}H]glycine overflow was -0.24 ± 0.09 and 1.85 ± 0.72% of content at normal and reduced Na^+ concentrations, respectively (n = 4). Values shown are the mean ± SEM of 4 experiments.

GABA release was an external Ca^{2+}-dependent process and the GABA reuptake inhibitor nipecotic acid potentiated this effect. In addition, slices from rat hippocampus were incubated with [^{3}H]glycine, superfused and the efflux of [^{3}H]glycine was measured in conditions where glycine reuptake was inhibited. Using this approach, we observed that (1) NMDA induced GABA release from the vesicular pool (2) kynurenic acid reduced the effect of NMDA on GABA release and glycine reversed this inhibition, (3) the GlyT1 inhibitor GDA did not affect glycine efflux, (4) in Na^+-deficient conditions, the outflow of glycine increased and electrical stimulation evoked glycine release.

Mechanism of NMDA Depolarization-Induced [^{3}H]GABA Release in Striatum. The selective glutamate receptor agonist NMDA dose-dependently enhanced the basal release of [^{3}H]GABA from superfused striatal slices of the rat. This effect of NMDA may be explained by inducing depolarization of cell membrane as monovalent and bivalent ions flow through the receptor-ion channel complex upon NMDA receptor stimulation (34,35). Besides Ca^{2+} entry through the NMDA receptor-ion channel, the increase in Na^+ influx secondarily leads to rise of intracellular Ca^{2+} concentration by opening of voltage-sensitive Ca^{2+} channels (36). The importance of the rise of $[Ca^{2+}]i$ concentration in the action of NMDA was shown by removing Ca^{2+} from the superfusion buffer which led to decrease of NMDA-induced [^{3}H]GABA release. These data strongly suggest that the NMDA depolarization-induced [^{3}H]GABA release in neostriatum is an external Ca^{2+}-dependent, vesicle-originated exocytotical process (37). Similarly to GABA release, the NMDA-induced dopamine and acetylcholine release were also dependent on external Ca^{2+} (38).

Experiments involved the GABA transporter inhibitor nipecotic acid further confirmed the vesicular origin of NMDA-induced GABA release. Nipecotic acid has higher affinity to GABA transport system than does GABA itself and it is also a substrate for GABA transporter (39,40). The fact, that nipecotic acid stimulates basal [^{3}H]GABA release, indicates that an active GABA transporter may be operative even in superfused striatal slices (29,41). It has been reported that nipecotic acid increases AMPA-induced as well as electrically induced [^{3}H]GABA release from striatum, conditions in which involvement of vesicular GABA pool has been demonstrated (33,42). Thus, our observation that nipecotic acid potentiated Ca^{2+}-dependent NMDA-induced [^{3}H]GABA release may also suggest that the pool which release is originated from is primarily vesicular and not cytoplasmic.

Contribution of Glycine$_B$ Binding Site in the Regulation of [^{3}H]GABA Release in the Striatum. It has been reported that in vitro addition of glycine to brain slice preparations does not generally influence NMDA receptor-mediated responses (43,44,45). This was also shown for NMDA-induced release of dopamine or acetylcholine in striatal slices (46). In the experiments of Garcia and coworkers (37), the olfactorial bulb tissues were previously depolarized with high K^+ concentration and glycine potentiated NMDA response in these conditions. We also found that glycine, in the absence of Mg^{2+}, failed to potentiate the effect of NMDA on [^{3}H]GABA release. These data strongly indicate that, at least in in vitro conditions, glycine$_B$ binding site of NMDA receptor may be saturated by endogenous glycine. This may explain why prior administration of the glycine$_B$ site antagonist kynurenic acid was required for demonstration of glycine stimulation. Kynurenic acid by itself failed to alter resting [^{3}H]GABA release suggesting that stimulation of NMDA receptor complex by glutamate released is necessary to evoke glycine release which then occupies glycine$_B$ site at a fully saturated rate.

Contribution of GlyT1 in the Regulation of [^{3}H]Glycine Efflux in the Hippocampus. To characterize the involvement of glycine transporter proteins, [^{3}H]glycine efflux was measured in hippocampal slice preparation in the present and absent of glycine reuptake inhibitors. Two glycine derivatives; GDA and

N-dodecylsarcosine showed glycine uptake inhibitory properties in synaptosomes prepared from hippocampus and the calculated IC_{50} value for GDA was in the same magnitude as was reported in the literature (22). Addition of GDA to superfused hippocampal slices was, however, without effect on [^{3}H]glycine outflow. Using a continuous superfusion, the inability of carrier inhibition is often found to influence spontaneous transmitter outflow. This may probably be explained by a washout effect since neurotransmitter transporters have been reported to be operative even in slice preparations (29,41). Whereas a large set of compounds compete with neurotransmitters for uptake, only a few of them possess releasing activity (47) and GDA may induce glycine uptake inhibition without release induction.

In Na^+-free buffer, [^{3}H]glycine efflux increased considerably which can be explained by either in terms of the blockade of Na^+-dependent transport into the cells or the release of glycine from cells via exchanger (48). This observation implies that uptake into the cells plays a significant role in removing glycine from extracellular fluid. When loaded with [^{3}H]glycine and superfused with buffer containing normal Na^+ concentration, efflux of radioactivity was observed from hippocampal slices. It was, however, rather surprising that stimulation of the hippocampus with high frequency failed to evoke elevation of basal [^{3}H]glycine outflow. This may indicate that glycine transport directed toward glial cells but this glia is unable to release glycine during electrical depolarization as their membrane cannot be excited by electrical pulses (48). Electrical stimulation effectively evoked [^{3}H]glycine release from hippocampus in which lowering extracellular Na^+ concentration led to inhibition of glycine transporters and this release may be originated from neural cells. It is possible to speculate therefore, that glycine uptake to glial cell and glycine release from neurons may occur simultaneously in excitable synapses.

Our view on operation of an excitable synapse is shown in Fig. 4. Glutamate released from nerve endings

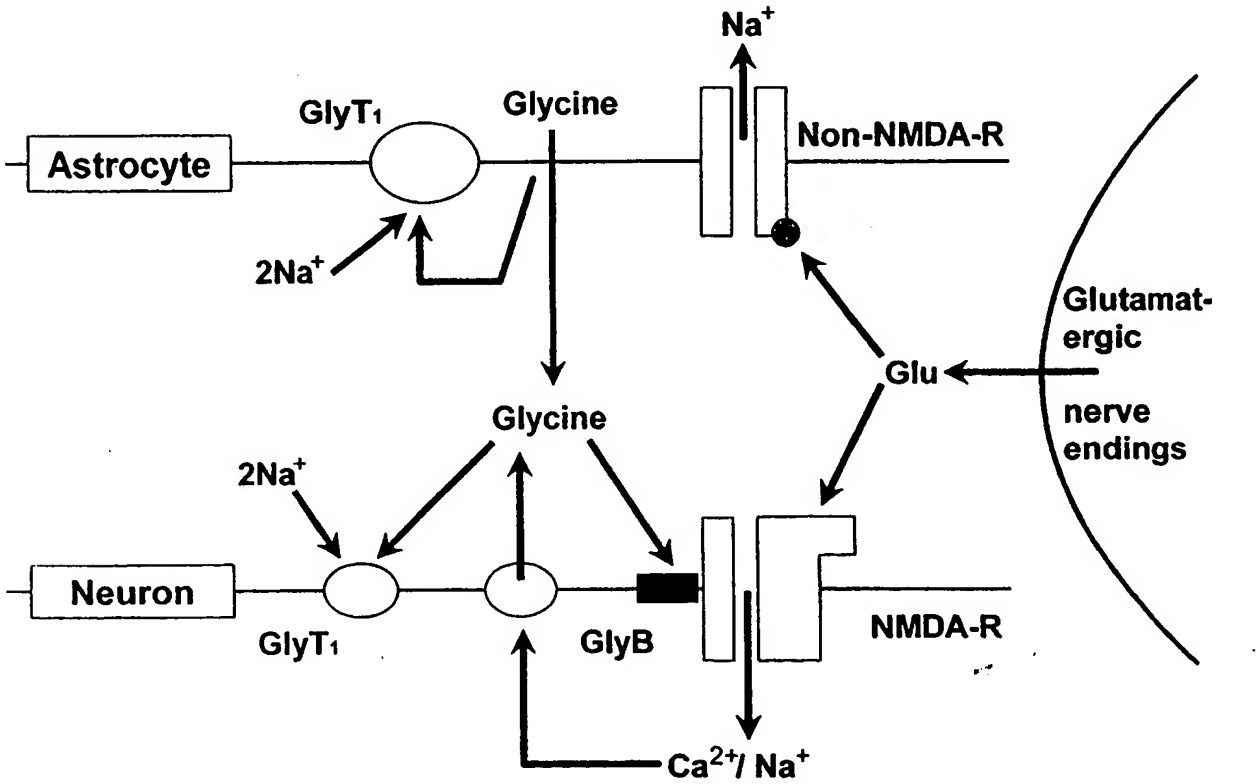

Fig. 4. Hypothetical model showing the role of glycine transporters in the regulation of glutamatergic neurotransmission. Glutamate released from nerve terminals binds to postsynaptic NMDA receptors and also to NMDA/non-NMDA receptors on glial cells. The latter event may lead to increase of glycine efflux from glia cells. Glycine together with glutamate then activates NMDA receptors, although the two cotransmitters bind to different sites. The release of glycine may also occur from postsynaptic neurons sensitive to electrical stimulation. Glycine concentration in the synaptic cleft is regulated by homotransporter on glial cell and by heterotransporter located in vicinity of postsynaptic NMDA receptor. Glycine transport proteins may be inhibited by uptake blockers which then lead to increased activity of NMDA receptors. Glu: glutamate, NMDA-R: NMDA receptor, non-NMDA-R: non-NMDA receptor, GlyB: glycine$_B$ binding site, GlyT$_1$: glycine transporter1.

activates postsynaptic NMDA receptors and the consequent ion channel opening assures influx of Na^+ and Ca^{2+} into the cells. Changes in intracellular ion milieu of neurons that possess NMDA receptors will then lead to release of glycine into the synaptic cleft. Activation of the powerful glycine transporter located in the neighboring astrocytes will reduce glycine levels in the synapse. Thus, neural release and glial uptake of glycine may occur simultaneously and the balance between the two processes will determine the rate of occupancy of $glycine_B$ sites on NMDA receptors.

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Glycine Tranporter-1 Blockade Potentiates NMDA-Mediated Responses in Rat Prefrontal Cortical Neurons In Vitro and In Vivo

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Chen, Long, Mark Muhlhauser, and Charles R. Yang. Glycine tranporter-1 blockade potentiates NMDA-mediated responses in rat prefrontal cortical neurons in vitro and in vivo. *J Neurophysiol* 89: 691–703, 2003. First published October 30, 2002; 10.1152/jn.00680.2002. The *N*-methyl-D-aspartate (NMDA) receptor (NMDA-R) has pivotal roles in neural development, learning, memory, and synaptic plasticity. Functional impairment of NMDA-R has been implicated in schizophrenia. NMDA-R activation requires glycine to act on the glycine-B (GlyB) site of the NMDA-R as an obligatory co-agonist with glutamate. Extracellular glycine near NMDA-R is regulated effectively by a glial glycine transporter (GlyT1). Using whole-cell voltage-clamp recordings in prefrontal cortex (PFC) slices, we have shown that exogenous GlyB site agonists glycine and D-serine, or a specific GlyT1 inhibitor *N*[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)-propyl]sarcosine (NFPS) in the presence of exogenous glycine (10 μM), potentiated synaptically evoked NMDA excitatory postsynaptic currents (EPSCs) in vitro. Furthermore, in urethan-anesthetized rats, microiontophoretic NMDA pulses excite single PFC neurons. When these responses were blocked by approximately 50% to approximately 90% on continuous iontophoretic application of the GlyB site, antagonist (+)HA-966, intravenous NFPS (5 mg/kg), or a GlyB site agonist D-serine (50 mg/kg iv) reversed this (+)HA-966 block. NFPS may elevate endogenous glycine levels sufficiently to displace (+)HA-966 from the GlyB sites of the NMDA-R, thus enabling reactivation of the NMDA-Rs by iontophoretic NMDA applications. D-Serine (50–100 mg/kg iv) or NFPS (1–2 mg/kg iv) alone also augmented NMDA-evoked excitatory responses. These data suggest that direct GlyB site stimulation by D-serine, or blockade of GLYT1 to elevate endogenous glycine to act on unsaturated GlyB sites on NMDA-Rs, potentiated NMDA-R-mediated firing responses in rat PFC. Hence, blockade of GlyT1 to elevate glycine near the NMDA-R may activate hypofunctional NMDA-R, which has been implicated to play a critical role in the pathophysiology of schizophrenia.

INTRODUCTION

Functional heteromeric *N*-methyl-D-aspartate (NMDA) receptors (NMDA-R), consisting of an obligatory combination of a NR1 subunit with any of the four NR2 (A–D) subunits (Cull-Candy et al. 2001; Monyer et al. 1992, 1994), are critically involved in neural development, synaptic plasticity, excitotoxicity, learning, and memory (Bliss and Collingridge 1993; Malenka and Nicoll 1999). The ionotropic NMDA-R possess multiple modulation sites on the receptor subunits: voltage-dependent Mg^{2+} block of the receptor channel pore (Mayer et al. 1984; Nowak et al. 1984), a strychnine-insensitive glycine-B (GlyB) site on the NR1 subunit, a phencyclidine (PCP) site on NR2 subunit, as well as the Zn^{2+}, polyamine, glycosylation, and polyamine sites (McBain and Mayer 1994). Extracellular glycine is an obligatory co-agonist for NMDA-R activation (Johnson and Asher 1987; Kleckner and Dingledine 1988; Parsons et al. 1998; Thomson 1990). Only when glycine binds to the GlyB site on the NR1 subunit, with glutamate to the glutamate-binding site on NR2 subunits, do single NMDA-R channels open (Anson et al. 1998; Currás and Pallotta 1996; Hirai et al. 1996; Laube et al. 1997). Functionally, this glycine binding allosterically influences NMDA-R to increase the recovery rate from receptor desensitization during synaptic activation (Benveniste et al. 1990; Lester et al. 1993; Mayer et al. 1989; Vyklicky et al. 1990).

A considerable debate centers on whether the GlyB site on the NMDA-R is saturated under physiological condition in vivo. Early in vitro studies suggested that endogenous glycine could have saturated the GlyB site on the NMDA receptor (Bashir et al. 1990; Fletcher and Lodge 1988; Kemp et al. 1988). Indeed, extracellular glycine concentration ($[glycine]_o$) is in the micromolar range in vivo, and the measured affinity of the GlyB site for glycine on NMDA-R is in the sub-micromolar range (K_i = 0.1–0.3 μM) (Baron et al. 1996; Grimwood et al. 1992). However, recent findings suggest that $[glycine]_o$ near the NMDA-R in the forebrain is efficiently regulated by a Na^+/Cl^--dependent, astroglial, high-capacity glycine transporter (GlyT) adjacent to the NMDA-R (Borowsky et al. 1993; Smith et al. 1992; Zafra et al. 1995). Hence, the transport actions of glycine transporter (GlyT1) and/or intracellular glycine sequestration may exceed the K_d of the glycine-binding site and help to rapidly keep $[glycine]_o$ near the NMDA-R to low levels (e.g., <1 μM) (Bergeron et al. 1998; Supplisson and Bergman 1997).

GlyTs belong to a superfamily of 12 *trans*-membrane domains, Na^+-dependent, neurotransmitter transporters. Two different genes, GlyT1 and GlyT2, encode GlyT. Transcription of GlyT1 gene resulted in more than or equal to three mRNA isoforms: GlyT1a, GlyT1b, GlyT1c, and with transcription of GlyT1a and GlyT1b mediated by alternative promoter usage (Adam et al. 1995; Borowsky and Hoffman 1998; Kim et al. 1994). While GlyT2 mRNAs are present in axonal terminals of glycinergic neurons and specifically regulate strychnine-sensitive glycinergic neurotransmission in brain stem and spinal cord, GlyT1 mRNAs are heterogeneously present in frontal cortex and hippocampus, as well as lower brain stem and spinal

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cord (Borowsky et al. 1993; Legendre 2001; Smith et al. 1992; Zafra et al. 1997). GlyT1 can remove glycine efficiently near the NMDA-R, as well as releasing glycine on changes in extracellular glycine and/or ionic composition (Berger et al. 1998; Fedele and Foster 1992; Herdon et al. 2001; Sakata et al. 1997; Supplisson and Bergman 1997). Saturation of the GlyB site by glycine in vivo may depend on the density of GlyT1, as well as the regional differences of local brain glycine levels (see Danyz and Parsons 1998; Wood 1995).

Dysfunction of the prefrontal cortex (PFC) and central NMDA-R plays a crucial role in the complex pathophysiology including severe cognitive deficits in schizophrenia (Breier 1999; Goldman-Rakic 1999; Javitt and Zurkin 1991; Tamminga 1998; Tsai and Coyle 2002; Weinberger and Berman 1996; Yang et al. 1999). One therapeutic strategy is to administer daily a large glycine dose (e.g., in grams per day, due to its poor CNS penetrance) (Oldendorf 1973), or GlyB site agonist D-serine, or a partial agonist cycloserine. These treatment approaches led to reported improvement of neuropsychiatric symptoms, presumably via potentiation of NMDA-R functions (Goff et al. 1999; Heresco-Levy and Javitt 1999; Javitt et al. 1994; Tsai et al. 1998, 1999).

Another way to elevate endogenous $[glycine]_o$ near NMDA-R is to block glycine uptake by GlyT1, although the question on whether GlyB site is saturated in PFC in vivo must first be clarified. The sarcosine derivative *N*[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)propyl]sarcosine (NFPS, also known as ALX-5407) represents a useful tool that has been shown to block glycine uptake by glial GlyT1 (K_i = 5 nM; IC_{50} = 0.03–0.22 μM) (Atkinson et al. 2001; Aubrey and Vandenberg 2001; Bergeron et al. 1998; Herdon et al. 2001) and to augment hippocampal NMDA-R-mediated synaptic responses in vitro (Bergeron et al. 1998). Although NFPS can elevate $[Glycine]_o$ levels in PFC and hippocampus in vivo (Atkinson et al. 2001; G. Nomikos and K. Johnson, personal communication), it is not known whether NMDA receptor functions can be potentiated in vivo. Since PFC is a key brain region where NMDA-R dysfunction in schizophrenia has been implicated (Tsai and Coyle 2002), the present electrophysiological study aims to determine whether stimulation of GlyB site by D-serine, glycine, or blockade of GlyT1 native to the PFC by NFPS will augment evoked NMDA excitatory postsynaptic currents (EPSCs) in PFC slices. In addition, by using the GlyB site antagonist (+)HA-966 to block NMDA-evoked firing in vivo, we also determined the site of action of D-serine and elevated $[glycine]_o$ following blockade of native GlyT1 by NFPS in vivo. Our data have provided key evidence to show that in vivo augmentation of endogenous $[Glycine]_o$ in PFC can lead to a potentiation of NMDA-R-mediated neuronal excitability. Preliminary results have been reported in an abstract form (Chen et al. 2001).

METHODS

Brain slice preparations

The experiments were performed in brain slices prepared from young adult (P25–35) male Sprague-Dawley rats. The euthanasia method used was approved by the Lilly Animal Use Committee, whose policies adhere closely with the U.S. *Public Health Service Policy on Humane Care and Use of Laboratory Animals* (PHS Policy) and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Guide). Following decapitation by a guillotine (using a plastic rat restrainer Decapicone, Braintree Scientific, FL), the brain was quickly removed and placed for 1 min in ice-cold oxygenated (95% O_2-5% CO_2) artificial cerebrospinal fluid (ACSF) containing the following (in mM): 124 $NaCl_2$, 26 $NaHCO_3$, 3.0 KCl, 0.5 $CaCl_2$, 4.0 $MgCl_2$, 0.4 ascorbic acid, 0.8 thiourea, 10 glucose. The temporal lobes of the cortex from both hemispheres were trimmed away, leaving the medial prelimbic PFC of both hemispheres. The prelimbic PFC corresponds to the region outlined in the stereotaxic atlas of Paxinos and Watson (1998) (A–P = 2.2–3.5 mm anterior to the bregma; D–V = 3–5 mm from the cortical surface; M–L = 0.8–0.9 mm from the midline). Bilateral coronal PFC slices (350-μm-thick) were then cut on a vibratome (Vibroslice, World Precision Instruments). After cutting, the slices were placed in warm (30°C) continuously oxygenated ACSF, containing the following (in mM): 124 NaCl, 26 $NaHCO_3$, 3 KCl, 2.0 $CaCl_2$, 1.3 $MgCl_2$, 10 glucose. After a 30-min incubation, the slices were cooled in the same ACSF to room temperature (22–23°C) for at least 1 h. A single slice was transferred to a submersion recording chamber (Warner Instrument) and electrophysiological recordings were made at 30–32°C. The temperature of the ACSF entering the recording chamber was rapidly heated to the preset temperature using an in-line heater (SH27B, Warner Instruments). The temperature of the perfusate (30–32°C) was maintained constant via an automatic feedback temperature controller (TC-324B, Warner Instruments).

Whole cell patch-clamp recordings

An upright Olympus BX50WI microscope equipped with differential interference contrast optics and infrared videoimaging system (DIC-IR, Hamamatsu C2400-07ER) was used to visualize neurons in slices. Layer V–VI PFC pyramidal neurons were easily recognizable via a 40× water-immersion lens by the pyramidal shape of their cell bodies and the presence of a long apical dendrite extending toward superficial layers. In some neurons, the morphology of single neurons from which recordings were made using biocytin (0.2%)-filled patch pipettes was confirmed by streptavidin–horseradish peroxidase staining of biocytin (Yang et al. 1996).

Whole cell patch-clamp techniques were used to study synaptic responses of layers V–VI pyramidal neurons in response to local layer V–VI stimulation in the prelimbic region of the PFC. Patch pipettes (3–5 MΩ) were fabricated from borosilicate tubing (1.5 mm OD, 1.1 mm ID) on a horizontal microelectrode puller (P-97, Sutter Instruments). The internal pipette solution contained the following (in mM): 100 potassium methyl sulfate, 60 sucrose, 10 HEPES, 1 EGTA, 2 $MgCl_2$, 2 Na_2ATP, 0.5 Tris–guanosine 5′-triphosphate (GTP), 10 Di-Na^+ phosphocreatine, pH was adjusted to 7.3 by KOH and had an osmolality of 285–295 mOsm.

Under voltage clamp, the current signal was amplified by an Axopatch 200B amplifier (Axon Instruments, Foster City). All signals were digitized with a 12 bit A/D converter (Digidata 1200B) and stored in the computer hard-drive for off-line analysis. Series resistance (10–20 MΩ after "break-in") was not compensated but was monitored periodically during the entire experiment. Recordings were terminated and the data are discarded if the series resistance changed by >10 MΩ.

In the voltage-clamp experiments, the slices were initially bathed with continuously oxygenated (95% O_2-5% CO_2) ACSF containing the following (in mM): 124 NaCl, 26 $NaHCO_3$, 3 KCl, 2.0 $CaCl_2$, 1.3 $MgCl_2$, 10 glucose. Once whole-cell recording was achieved, the media were switched to an ACSF solution with low Mg^{2+} (0.1 mM) and high Ca^{2+} (3.6 mM to maintain divalent cation concentrations; see Bergeron et al. 1998) containing LY300168 [50 μM, a noncompetitive selective antagonist of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, formerly known as GYKI 53655] and SCH50911 [10 μM, a selective antagonist for γ-aminobutyric acid-B ($GABA_B$) receptor] to pharmacologically isolate the

NMDA EPSCs. Alteration of $[glycine]_o$ concentration for each series of experiments is specified in the appropriate sections of the text. Neurons were voltage clamped at a V_{Hold} of −75 to −80 mV. At the beginning of the experiments, whole-cell evoked NMDA EPSCs were examined at different holding voltages (from −100 to −50 mV). Only cells with little or no evoked γ-aminobutyric acid-A ($GABA_A$) outward current were selected. At the end of experiments (80% of all experiments), the competitive NMDA antagonist D-2-amino-5-phosphonovaleric acid (APV; 50 μM) was added to ensure that all inward currents evoked and potentiated were NMDA receptor mediated (see Fig. 1 and 3*B*).

Synaptic stimulations

Electrical stimulation was delivered via a concentric bipolar metal-stimulating electrode (MCE-100X, David Korpf) placed in layer V, approximately 200–300 μm from the adjacent recorded neuron to activate local afferents synaptically. Programmed monophasic square-pulses (0.1 ms, 50–200 μA, at 30-s inter-stimulus intervals) were delivered via a Master-8 programmable pulse-generator to an optically isolated stimulator (Isoflex, A.M.P.I., Israel).

Extracellular single-unit recording with microiontophoresis

Male Sprague-Dawley rats (250–320 g, >P45) were anesthetized with urethane (1.5 g/kg, ip) and mounted in a stereotaxic frame (Stoelting, Harvard Apparatus). Core temperature was monitored by a rectal probe and maintained at 37°C by a heating pad (Federick Haer, Brunswick, NJ). Burr holes were drilled through the skull over the PFC (stereotaxic coordinates for the PFC : A–P = 2.7–3.0 mm anterior to the bregma, L–M = 0.8–1.0 mm, D–V = 2.0–3.5 mm from the cortical surface; Paxinos and Watson 1998). A venous catheter, made of PE-10 tubing, was inserted into the jugular vein for intravenous syringe infusion of drugs.

Conventional extracellular single-unit recordings with iontophoresis were made using five-barrel glass micropipettes. The multi-barrel pipette blanks (World Precision Instruments) were pulled by a vertical Narishige pipette puller (PE-2, Narishige, Tokyo, Japan). The recording center barrel was filled with 0.5% sodium acetate in 2% Pontamine Sky blue mixed with bicuculline methiode (0.5 μM). A slow leak of bicuculline was used to partially block local $GABA_A$ receptor-mediated tonic inhibitory responses (Gigg et al. 1994). The side-barrels were filled with NMDA (2–20 mM, pH 8; Sigma, St. Louis, MO), the Gly-B site antagonist (+)HA-966 (0.2 mM, pH 4), and NaCl (200 mM, for current balancing). The electrode was advanced by a single-axis hydraulic micromanipulator (MHW-40-1, Narishige) mounted onto the stereotaxic frame.

Extracellular single-unit activity was amplified by a Xcell-3 Plus amplifier (Frederick Haer). Amplified (gain: 100,000×; low-pass filter at 5 kHz, high-pass filter at 500 Hz) single-unit activity was isolated using a window discriminator (model 74-60-3, Frederick Haer). The output signals from the window discriminator were digitized and multiplexed by an A/D converter (1401 mini, Cambridge Electronics Design, CED, Cambridge, UK) and were sampled at 10 kHz by a PC-based computer using Spike 2 software (Version 4, Cambridge Electronics Design). Programmed NMDA pulses (−20 to −40 nA) were iontophoretically applied repeatedly (Dagan 6400, Dagan) for 10–25 s every 45–60 s to evoke firing of single PFC

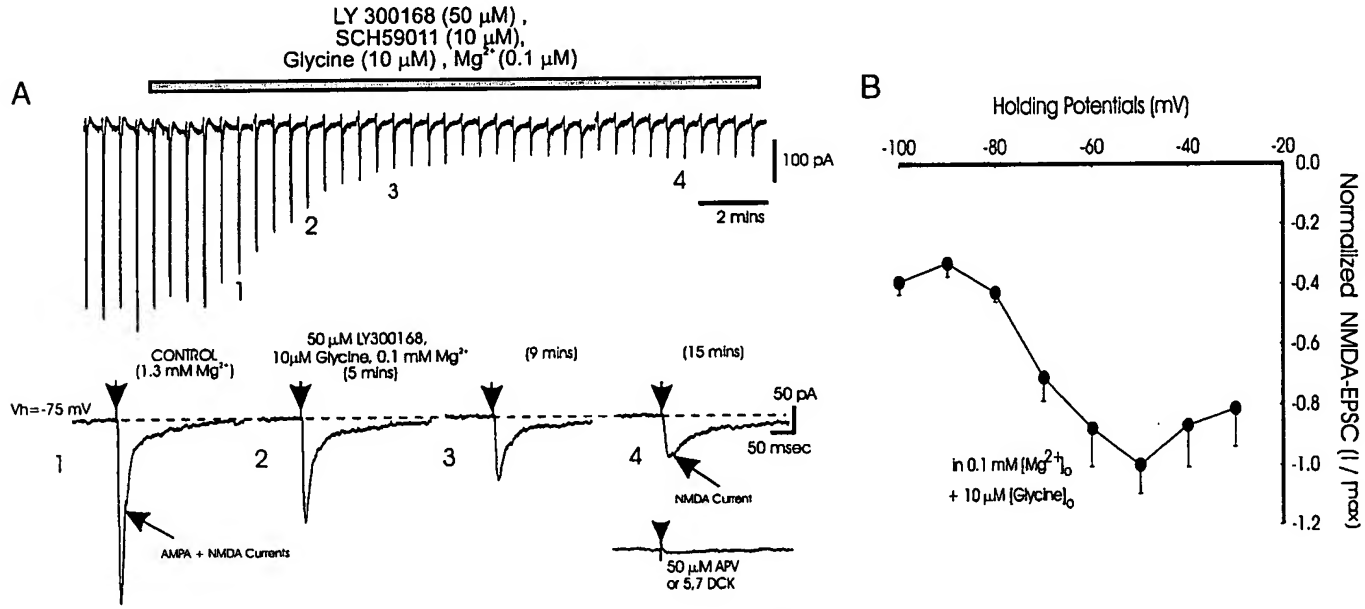

FIG. 1. Pharmacological isolation of synaptically evoked *N*-methyl-D-aspartate (NMDA) excitatory postsynaptic current (EPSC). *A*: evoked synaptic responses over time showing the gradual pharmacological isolation of the NMDA component of the evoked EPSC. Electrical stimulation was delivered once every 30 s (i.e., at 0.033 Hz) to layer V–VI adjacent to the recorded pyramidal neuron in prefrontal cortex (PFC). The gray bar on top indicates the time when a mixture of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist LY300168 (50 μM), glycine (10 μM), and γ-aminobutyric acid-B ($GABA_B$) antagonist SCH59011 (10 μM) were bath-applied in perfusate that contained low Mg^{2+} (0.1 mM) and high Ca^{2+} (3.6 mM). In the early period of perfusion (1), synaptic responses consisted of both a large-amplitude AMPA receptor-mediated component and a late slow NMDA component in the EPSC. 2–3: A gradual blockade of the AMPA receptor component of the EPSC at 5 and 9 min of perfusion with the above drug cocktail. 4: (*top*) Pharmacologically isolated NMDA EPSC. (*bottom*) Application of competitive D-2-amino-5-phosphonovaleric acid (APV; 50 μM) or noncompetitive [5,7-dichlorokyneurinic acid (5,7-DCK) 50 μM] NMDA receptor antagonist completely blocked the EPSC, suggesting that it is an NMDA EPSC. *B*: normalized NMDA EPSC (integrated area) evoked at different holding potentials in the presence of low Mg^{2+} and $[glycine]_o$ (10 μM) (filled circles). Note that the voltage-dependent augmentation of NMDA EPSC was enhanced at all holding potentials in low $Mg^{2+}/[glycine]_o$ condition. In the graphical plot the peak NMDA EPSC responses were observed at a $V_m \approx -50$ mV and displays a region of negative slope conductance. We chose a holding potential of −75 to −80 mV for evoking our control NMDA EPSC in this study.

neurons. (+)HA-966 was iontophoretically applied (30–80 nA) continuously to block the NMDA-evoked firing responses. An additional barrel in the five-barrel pipette contains saline (0.9% NaCl). Automatic ejection of currents in opposite polarity with respect to drug ejection current applied was made for current balancing to eliminate possible current artifacts.

To verify the position of the microelectrode, DC current (10 μA, for 15 min) was delivered to iontophorese Pontamine Sky blue through the recording electrode to mark the recording site. The animal was then perfused transcardially with saline, followed by buffered formalin. Brain sections (70 μm) containing the PFC were cut using a freezing microtome (Leitz, Germany) and the sections were washed, dehydrated with alcohol, and stained with cresyl violet to permit examination of the recording sites.

Drug applications

For in vitro brain slice experiments, all drugs used were bath-applied by gravity. Stock solutions of APV, bicuculline, 5,7-dichlorokyneurinc (5,7-DCK), (+)HA-966, and D-serine were prepared in de-ionized water. All other drugs including the selective AMPA antagonist LY300168, GlyT1 inhibitor NFPS, were dissolved in DMSO and stored as frozen aliquots at −20°C and diluted to appropriate concentrations in ACSF for slice perfusion.

For in vivo experiments, NFPS was first dissolved in 200 μl ethanol. An appropriate amount of a hydroxy-β cyclodextran solution (50%, HBC; Sigma) was added and sonicated briefly. Deionized water was then added to make a final concentration of HBC to 15%. Intravenous administration of drug vehicle alone showed no change in NMDA-evoked firing.

Data analyses

For in vitro brain slice experiments, the integrated areas and amplitudes of evoked EPSCs were measured using pClamp 8.0 software (Axon Instruments). The decay time constant of the averaged NMDA EPSC was fitted for two exponentials from the peak of the EPSC to the end of the trace using standard exponential fitting formula in pClamp 8.0 (Axon Instruments)

$$[f(t) = \sum_{i=1}^{n} A_i e^{-t/\tau_i} + C]$$

where i is the current as a function of time (t), A_i and τ_i are the amplitude and time constant of each component of the current, respectively, and C is the constant y-offset for each component i. The NMDA-EPSC trace recorded following different doses of GlyT inhibitor NFPS, glycine, or D-serine was scaled to the same peak amplitude of the predrug control EPSC. All group data were presented as mean ± standard error (SE). Analysis of variance (ANOVA) and post hoc Dunnett's test was used to compare differences between group mean data with control group mean. Differences between control and experimental responses with $P < 0.05$ were deemed significant. Student's t-test was used for group comparison to determine exogenous glycine and D-serine effects on evoked NMDA EPSCs.

For in vivo iontophoretic data, the control NMDA-evoked firing measured from five to six repeated stable baseline responses were averaged and analyzed using Spike 2 software (version 4, CED). Following systemic drug injections, the mean firing rates evoked by NMDA application that consist of responses at 20% or greater than baseline NMDA-evoked responses were taken for comparison with the baseline mean data. Group data comparisons were made using one-way ANOVA, followed by post hoc Dunnett's test (GraphPad Prism Software).

RESULTS

In vitro experiments

CHARACTERIZATION OF SYNAPTICALLY EVOKED NMDA-EPSC. Electrical stimulation of layer V–VI synaptically evoked a mixed EPSC in layer V–VI pyramidal neurons. The EPSC was dominated by a large, fast AMPA receptor component. Bath application of the drug cocktail containing AMPA and $GABA_B$ antagonists in low $[Mg^{2+}]_o$ superfusate resulted in a gradual blockade over time of the prominent AMPA EPSC. This was followed by a slow emergence of the NMDA EPSC, which was pharmacologically confirmed by its sensitivity to blockade with the competitive NMDA antagonist APV (50 μM, $n = 3$), or the GlyB site NMDA antagonist 5,7-DCK (50 μM, $n = 3$) (Fig. 1). This suggests that the evoked EPSC is NMDA receptor mediated.

By varying the steady-state holding potentials prior to each stimulation, we have examined the voltage-dependence of the evoked NMDA EPSC. Low $[Mg^{2+}]_o$ (0.1 mM) perfusate in the presence of LY300168 and SCH52911 was used to isolate the NMDA EPSC. In low $[Mg^{2+}]_o$, elevating $[glycine]_o$ (10 μM) potentiated the NMDA EPSC integrated areas (Fig. 1*B*). The increase in evoked NMDA EPSC began at approximately −75 mV and gradually, the evoked inward current increased with more positive holding potentials to a maximal current evoked at −50 mV. There was a voltage-dependent reduction of the evoked NMDA EPSC with holding potentials more positive than −50 mV (Fig. 1*B*). Furthermore, the selected holding potentials of −75 to −80 mV (for recording NMDA EPSCs in low $[Mg^{2+}]_o$) are far from the activation voltage whereby the evoked EPSC could trigger dendritic Ca^{2+} current (Seamans et al. 1997), which can obscure the synaptic current.

The decay time constants of the NMDA EPSC represent a complex interaction of at least four factors. These factors include the following: slow dissociation of glutamate from the NMDA receptor (deactivation) (Hestrin et al. 1990; Lester and Jahr 1992), intrinsic channel kinetics (periodic cluster bursts of channel opening and closing in the presence of glutamate binding on the receptor) (D'Angelo et al. 1990, 1994; Gibb and Colquhoun 1991; Lester et al. 1990), intrinsic properties of the combined NMDA receptor subunits (Cull-Candy et al. 2001; Monyer et al. 1994; Vinci et al. 1998), and Ca^{2+}-mediated receptor desensitization (Clark et al. 1990; Lerma et al. 1990; McBain and Mayer 1994; Tong et al. 1995; Zilberter et al. 1991). The decay time-constant (tau, τ) of the NMDA-EPSC in PFC neurons could be fitted by two exponentials (D'Angelo et al. 1990, 1994): a fast, and a slow component, typically found in >P22-day-old rats as used in this study. The mean decay time of the slow τ reduces with age and this may be due to an age-dependent switch of the NMDA receptor subunit from NR2B to NR2A (Carmignoto and Vicini 1992; Flint et al. 1997; Hestrin 1992; Kew et al. 1998). In the present experiments, the fast component of the decay had a mean τ_{Fast} of 43.3 ± 2.7 ms (range: 33–52 ms), whereas the slow component had a mean τ_{Slow} of 276.8 ± 26.3 ms (range: 197–345 ms) ($n = 7$ analyzed).

BOTH GLYCINE AND D-SERINE DOSE-DEPENDENTLY POTENTIATED SYNAPTICALLY EVOKED NMDA EPSC. Next, incremental doses of GlyB agonists glycine or D-serine were bath-applied to determine the extent to which these amino acids potentiate the evoked NMDA-EPSCs. Increasing the concentration of gly-

cine and D-serine (from 0.1 to 100 μM) increased both the peak amplitude, as well as the integrated area [glycine: $F(3,28) = 3$, $P < 0.05$; D-serine : $F(3,22) = 14$, $P < 0.001$] of the NMDA EPSCs (Fig. 2, *A* and *B*). Group data summarized in Fig. 2*C* show that at 0.1, 1 μM D-serine induced a significantly greater potentiation of NMDA EPSC integrated area compared with that induced by glycine. However, at 10 and 100 μM D-serine and glycine, there was a considerable cell-to-cell variation in the NMDA EPSC potentiation effects, e.g., potentiation by glycine at 100 μM was in the range of 17–127%, while potentiation by D-serine at 100 μM was in the range of 20–190%. These large variations of NMDA EPSC modulation by the GlyB site agonists suggest a heterogeneous degree of GlyB site saturation perhaps due to different NR subunit combinations. Although each of the GlyB site agonist potentiated individual NMDA EPSC significantly, the potentiation of the NMDA EPSC by glycine versus D-serine (at 10 and 100 μM) did not reach statistical significance ($P > 0.05$).

One action of glycine is to accelerate the recovery of NMDA receptors from desensitization (Benveniste et al. 1990; Lerma et al. 1990; Mayer et al. 1989; Vyklicky 1993). As a result, glycine lengthens the duration of the decay time constants (τ) (Fig. 2*B*, *top*) of the evoked NMDA EPSCs. However, we found that glycine at 1, 10, and 100 μM exerted an inverted-"U" dose-response profile for the NMDA EPSC in PFC slices (Fig. 2*D*, *right*). While there was no overall change in the mean τ_{Fast} at any dose of $[glycine]_o$, the τ_{Slow} was significantly ($P < 0.02$) enhanced by glycine only at 10 μM (control = 182 ± 6.2 ms; 10 μM glycine = 274 ± 34.1 ms; $F(3,41) = 4.58$; $P < 0.01$; Dunnett's test, $P < 0.01$). A further increase of $[glycine]_o$ to 100 μM did not further increase the τ_{Slow}. Hence, the mean τ_{Slow} at 100 μM glycine did not differ from the control value ($P > 0.05$).

The GlyB site agonist D-serine (Brugger et al. 1990) potentiated the peak amplitude and τ_{Slow} of the NMDA-EPSCs in individual neurons (Fig. 2*B*), thus yielding a net dose-dependent increase in the integrated area (Fig. 2*C*). In some individual PFC neurons, although D-serine showed a dose-dependent

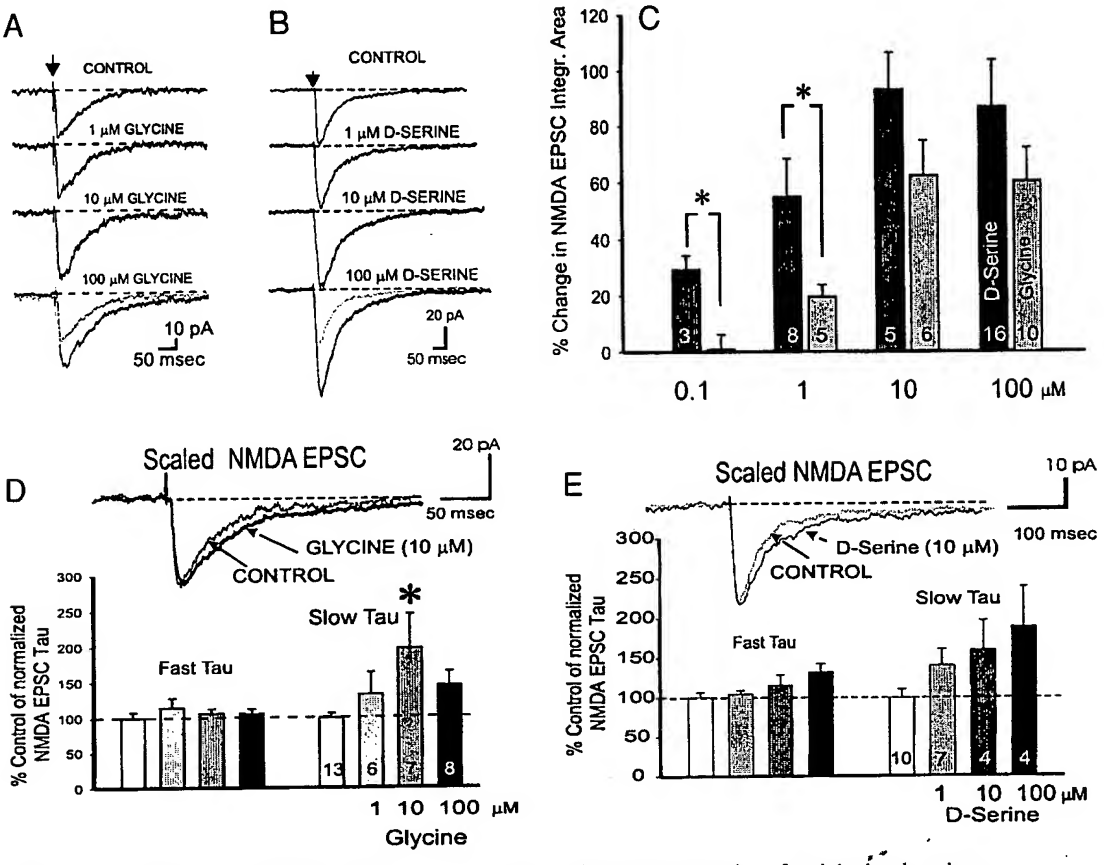

FIG. 2. Dose-dependent potentiation of the NMDA EPSC by glycine and D-serine and prolongation of mainly the slow decay time constant of the NMDA EPSC. *A–C*: increase of extracellular glycine (*A*), D-serine (*B*) dose dependently augments the peak and integrated area (*C*) of the evoked NMDA EPSCs in a PFC neuron (*$P < 0.05$; **$P < 0.005$). The superimposed gray traces in the last trace of *A* and *B* are control traces that are used for comparison. Control traces were obtained in nominally glycine-free [<100 nM from high-performance liquid chromatography (HPLC) analysis] ACSF. *D*: NMDA EPSC decay time constant typically show 2 components (fast and slow τ). There was no change in the fast τ with elevation of extracellular glycine concentrations. Increased concentration of extracellular glycine increases the slow $\tau \leq 10$ μM compared with the control (*$P < 0.05$). However, at 100 μM, there was no change in the slow τ with respect to the control. *Inset*: average trace of the NMDA EPSC in control and in glycine (10 μM). The traces are scaled to be the same as the control peak. Glycine moderately increases the slow decay time constant of the NMDA EPSC. *E*: in individual neurons an increase concentration of extracellular D-serine also augments the slow component of the decay time constant of the NMDA EPSCs but there is no effect on the fast decay time constant. However, group data analysis by analysis of variance (ANOVA) shows no dose-dependent change in the NMDA EPSC τ_{Slow} with incremental dose of D-serine. *Inset*: average trace of the NMDA EPSC in control and in D-serine (10 μM). The traces are scaled to be the same as the control peak and show that D-serine (10 μM) induced a moderate increase in the τ_{Slow} of the NMDA EPSC.

trend in enhancing τ_{Slow} of the NMDA EPSC (Fig. 2*D*), ANOVA test [$F(3,21) = 0.8$; $P = 0.5$] applied to group data failed to show a statistical dose-dependent change in the τ_{Slow} of the NMDA EPSC ($P > 0.05$; Fig. 2*E*). Similar to glycine, D-serine at 1, 10, and 100 μM also did not change the τ_{Fast} of the evoked NMDA-EPSC (Fig. 2*E*).

GlyT1 inhibitor potentiates synaptically evoked NMDA current in PFC neurons in vitro

In the absence of added extracellular glycine ($\leq$100 nM glycine present in the solution), NMDA-EPSCs were also evoked by electrical stimulation of the local afferents. This suggests that a trace amount of glycine is likely to be present in the tissue and/or the perfusate (as trace contaminant or metabolic product). When the GlyT1 inhibitor NFPS was bath-applied (0.01 μM), it moderately potentiated the evoked NMDA EPSC ($\approx$15%). However, further increases in the concentration of NFPS (from 0.1 to 10 μM) failed to cause further increases in the evoked NMDA EPSCs, suggesting that the effects of glycine on NMDA EPSC was at its maximum when there was no added extracellular glycine present in the media.

Since extracellular glycine levels in the PFC in vivo are approximately 10 μM (Hashimoto and Oka 1997), we then included 10 μM glycine in our low Mg^{2+} perfusate in the experiments to determine the effects of GlyT inhibitor NFPS on evoked NMDA EPSCs. Application of NFPS (from 0.01 to 10 μM) dose-dependently augmented the integrated area of the evoked NMDA-EPSCs (Fig. 3, *B* and *C*). Group data analysis of the decay τ of the NMDA EPSC shows that similar to glycine and D-serine, NFPS did not change the mean fast decay τ of the NMDA EPSC. Group analyses of the slow decay τ show that NFPS $\leq$1 μM showed a trend in causing a dose-dependent lengthening of the NMDA EPSC [$F(4,31) = 3.43$, $P < 0.01$]. Only at 1 μM did NFPS show a statistically significant increase of the slow decay τ with respect to the control (Dunnett's test, $P < 0.01$). Increasing the NFPS concentration to 10 μM (with 10 μM glycine) had no effect on the slow decay τ of the NMDA EPSC.

To rule out the possibility that NFPS might potentiate the NMDA EPSC via other mechanisms, we have conducted an additional experiment. In the presence of a saturating concentration of GlyB site agonist D-serine (100 μM) that is not normally transported by GlyT1 (Broer et al. 1990; Ribeiro et al. 2002; Schell et al. 1995, 1997; Snyder and Ferris 2000; Snyder and Kim 2000; Supplisson and Bergman 1997), the fully potentiated NMDA EPSC could not be further changed by the GlyT1 inhibitor NFPS (in 10 μM glycine) (Fig. 3*E*). This result suggests that NFPS did not have any additional unanticipated properties on NMDA EPSC.

It is difficult to absolutely rule out an increase in NMDA EPSC decay time constant by D-serine, glycine, or NFPS may also be due to a possible degraded voltage control under voltage-clamp when the EPSC has been greatly increased. However, activation of the GlyB site of the NMDA receptor by glycine or D-serine has been shown to prolong the decay kinetics of the NMDA EPSC or miniature EPSC, and likewise, the decay time constant of NMDA EPSC can be reduced by the GlyB site antagonist HA966 (e.g., Berger et al. 1998; Lester et al. 1993). Our findings are consistent with the findings from these studies.

In vivo experiments

EFFECTS OF D-SERINE AND NFPS ON IONTOPHORETIC NMDA-EVOKED EXCITATORY RESPONSES IN PFC NEURONS IN VIVO. Systemic injection of the GlyT inhibitor NFPS (1–2 mg/kg iv) significantly enhanced the NMDA-evoked spike firing (+76 $\pm$ 10%; $P < 0.05$, $n = 6$; Fig. 4, *A*--*D*) in single PFC neurons in vivo (Fig. 4). Since NFPS does not interact with NMDA-R directly (e.g., NFPS does not displace MDL105519 or MK-801 binding of the glycine, or the channel pore sites, respectively, of the NMDA receptor, Bergeron et al. 1998; D. Calligaro, personal communication), the most plausible explanation would be that NFPS blocked the GlyT1 to enable sufficient glycine accumulate near NMDA-R to potentiate the NMDA-evoked firing. This finding may further support the suggestion that the GlyB site of the NMDA-R is not saturated in PFC.

D-Serine is moderately better than glycine in penetrating the blood-brain barrier when administered systemically (Oldendorf 1973). In our in vivo studies, we tested whether the GlyB site of the NMDA receptor in the PFC was saturated by using the GlyB site agonist D-serine to probe at this issue. Iontophoretically application of NMDA (−20 to −60 nA) at an inter-application interval of approximately 1 min reliably excited single PFC neurons. Following acquiring a stable baseline of the NMDA-evoked firing responses, we administered D-serine (50–100 mg/kg iv) (Fig. 5*A*). Over a short period of time (10–15 min), the same NMDA iontophoretic pulses now elicited a significantly greater (+89.77 $\pm$ 8.75%; $P < 0.05$) number of spikes in these same neurons ($n = 6$). Since glycine and D-serine interact at the same GlyB site on the NMDA receptor, the enhancement of NMDA-evoked firing response by D-serine suggests that the GlyB site on the NMDA receptor, similar to that in visual cortex and hippocampus (Czepita et al. 1996; Dalkara et al. 1992; Mizutani et al. 1991; Salt 1989), is not saturated by endogenous glycine in rat PFC in vivo (Figs. 4 and 5).

D-SERINE OR GLYT1 INHIBITOR REVERSES THE BLOCKADE OF GLYB SITE OF NMDA RECEPTOR BY (+)HA-966 IN PFC NEURONS IN VIVO. To further determine how elevation of extracellular glycine may enable the endogenous glycine to interact with the GlyB site of the NMDA receptor and potentiate NMDA-evoked spike firing, we continuously applied iontophoretically a selective GlyB antagonist (+)HA-966 (Foster and Kemp 1989) to block the NMDA-evoked firing response first. We then administered D-serine to compete with HA-966 at the GlyB sites of the NMDA receptor, or the GlyT1 inhibitor NFPS to block GlyT1 (Atkinson et al. 2001).

In separate series of experiments using continuous iontophoretic application of (+)HA-966, we aimed to block the NMDA-evoked firing responses by $\approx$50%, or by $\approx$80% to determine the ability of the GlyB site agonist D-serine or NFPS to reverse the two levels of NMDA-R blockade. After achieving steady-state blockade of the NMDA evoked responses, we injected intravenous D-serine (50 mg/kg) or NFPS (5 mg/kg). In the group of PFC neurons with a partial (52 $\pm$ 15.6% of control) blockade of the NMDA-evoked responses, both D-serine (87.5 $\pm$ 5.5% of control, $P < 0.05$; Fig. 5*B*) or NFPS (113.8 $\pm$ 14.4% of control; $P < 0.05$; Fig. 6, *A*–*C*) signifi-

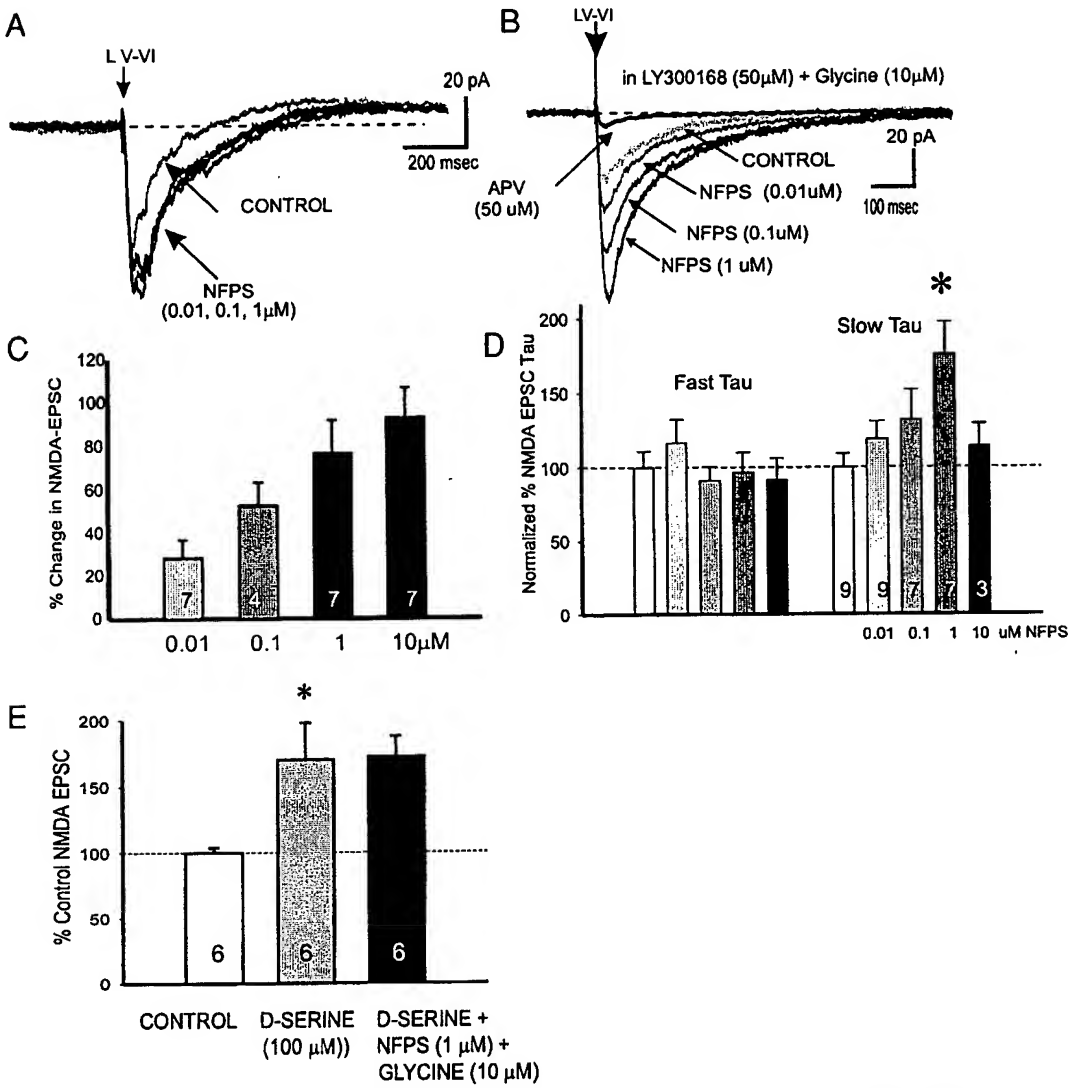

FIG. 3. *N*[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)propyl]sarcosine (NFPS) dose-dependently augment evoked NMDA EPSC in the absence and presence of added extracellular glycine (10 μM). *A*: NMDA EPSC traces evoked in no added extracellular glycine. NFPS (0.01 μM) augmented the NMDA EPSC. Increasing the concentration of NFPS to higher than 0.01 μM caused no further augmentation of the synaptic current. This suggests that NFPS can block endogenous glycine transport. Higher concentration of the glycine transporter (GlyT) inhibitor failed to cause further augmentation perhaps due to absence of transportable extracellular glycine. *B*: in the presence of added extracellular glycine (10 μM), NFPS dose-dependently potentiated the NMDA EPSCs. At the end of the experiment, application of the competitive NMDA antagonist APV (50 μM) blocked the evoked NMDA EPSC by 95% with a small residue (approximately 9 pA) inward current still remained. *C*: graphical plot showing the dose-dependent percentage increase from control of the NMDA EPSC by NFPS (in the presence of 10 μM extracellular glycine). *D*: analyses of the fast and slow component of the decay time constant of the NMDA EPSC show that there was no change in the τ_{Fast}. Although there was a trend for a dose-dependent increase in the slow component of the decay time constant, group comparisons showed that only at 1 μM that NFPS augmented NMDA EPSC τ_{Slow}. Further increase of NFPS to 10 μM failed to enhance the τ_{Slow} further. Instead, the τ_{Slow} dropped down to control value, just like the NMDA EPSC τ_{Slow} response to glycine at high dose (e.g., 100 μM, Fig. 2*D*). *E*: following a maximal dose of D-serine (100 μM) that potentiated the NMDA EPSC maximally, further addition of the GlyT1 inhibitor NFPS (1 μM, in the presence of 10 μM glycine) failed to cause any further changes in the fully potentiated NMDA EPSC. This suggests that when the glycine-B (GlyB) site on the NMDA receptor is fully saturated by D-serine (which is nontransportable by GlyT1), no additional effects of NFPS on NMDA receptor are achievable.

cantly reversed the blockade ($n = 6$ in each group). In the other group of PFC neurons with a greater blockade of the NMDA-evoked response (down to 23.86 ± 3% of control) by continuous iontophoretic application of (+)HA-966, intravenous D-serine (50 mg/kg iv) reversed the blockade to only 48 ± 7.5% of control while only NFPS (5 mg/kg iv) was able to reverse significantly ($P < 0.05$) the blockade to 60.3 ± 16% of control. These findings suggest that, while D-serine directly competes with (+)HA-966 at the GlyB site to restore NMDA evoked responses, NFPS may have enhanced extracellular glycine levels sufficiently to displace (+)HA-966 and hence restore NMDA-evoked firing responses.

DISCUSSION

Evoked NMDA-EPSCs were potentiated dose-dependently by GlyB site agonist glycine and D-serine, and by the GlyT inhibitor NFPS (in the presence of 10 μM extracellular gly-

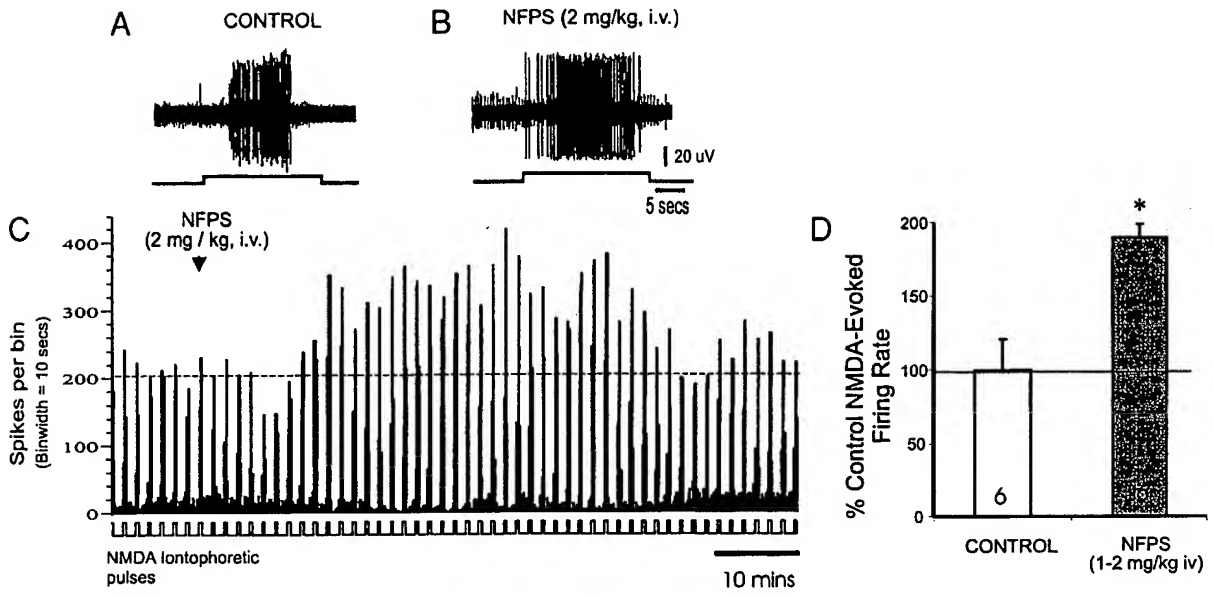

FIG. 4. The GlyT1 inhibitor NFPS potentiated NMDA-evoked firing of single PFC neurons in vivo. *A*: continuous voltage trace illustrating spike firing evoked by direct iontophoretic application of NMDA onto the PFC neuron. *B*: the same NMDA-evoked firing was enhanced 15 min following intravenous administration of NFPS (accumulative dose of 2 mg/kg). *C*: frequency-time histogram (binwidth = 10 s) showing a gradual enhancement of the NMDA-evoked firing following intravenous administration of NFPS. Note that the same NMDA iontophoretic pulses (−45 nA, 7 s applied iontophoretically every minute) were applied throughout the entire experiment. *D*: histograms summarizing the group data and showing a significant enhancement of the NMDA-evoked firing following NFPS administrations. $*P < 0.05$.

cine) in vitro. In vivo electrophysiological data show that excitatory responses to microiontophoretic application of NMDA were potentiated by intravenous administration of the GlyT inhibitor NFPS alone, or by the GlyB site agonist D-serine. Partial blockade of the GlyB site by continuous (+)HA-966 was reversed by intravenous D-serine or NFPS. Some of

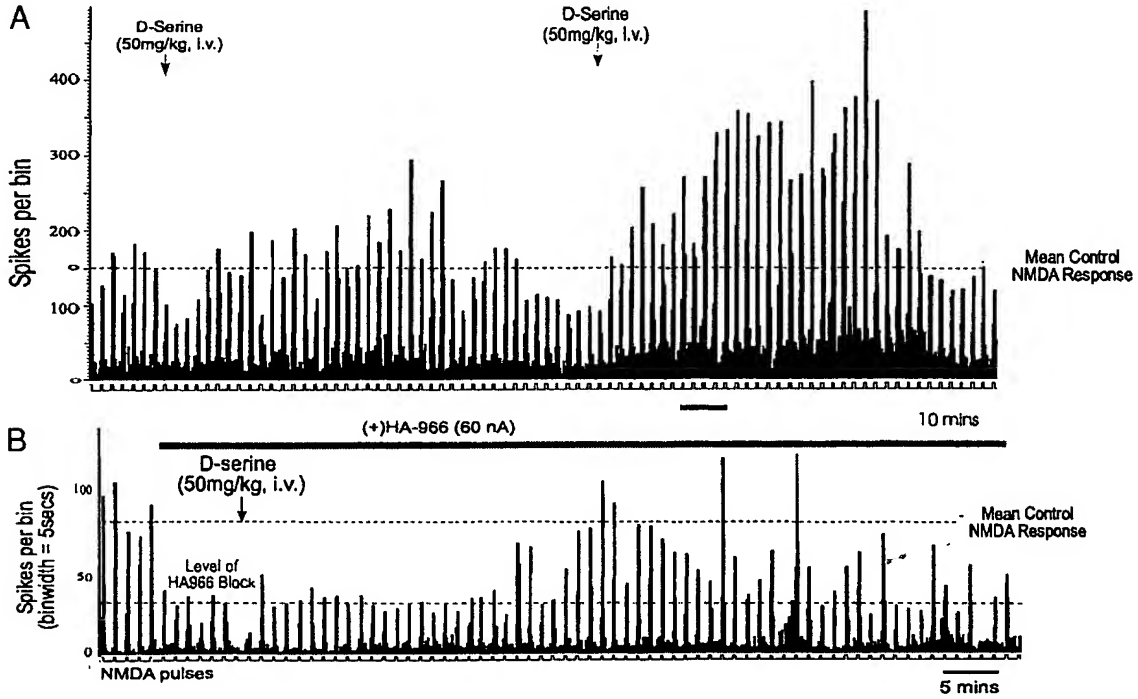

FIG. 5. The GlyB agonist D-serine augmented NMDA-evoked firing in PFC neuron and reversed partial blockade of the NMDA-evoked firing by iontophoretic application of the GlyB site NMDA antagonist HA-966. *A*: frequency-time histogram showing dose-dependent potentiation of NMDA-mediated firing responses in a PFC neuron. Binwidth = 5 s. In this PFC neuron intravenous administration of 50 mg/kg of D-serine induced a 45.3% increase in NMDA-evoked firing. An additional 50 mg/kg D-serine (= accumulated dose of 100 mg/kg) induced a 114% increase in NMDA-evoked firing. *B*: iontophoretic NMDA pulse evoked firing in a PFC neuron was blockaded partially by continuous iontophoretic application of (+)HA-966 (60 nA). Intravenous D-serine (50 mg/kg) was able to reverse the partial blockade of the NMDA-evoked firing, suggesting that D-serine acts by competing with the HA-966 at the unsaturated GlyB site of the NMDA receptor in PFC neurons in vivo.

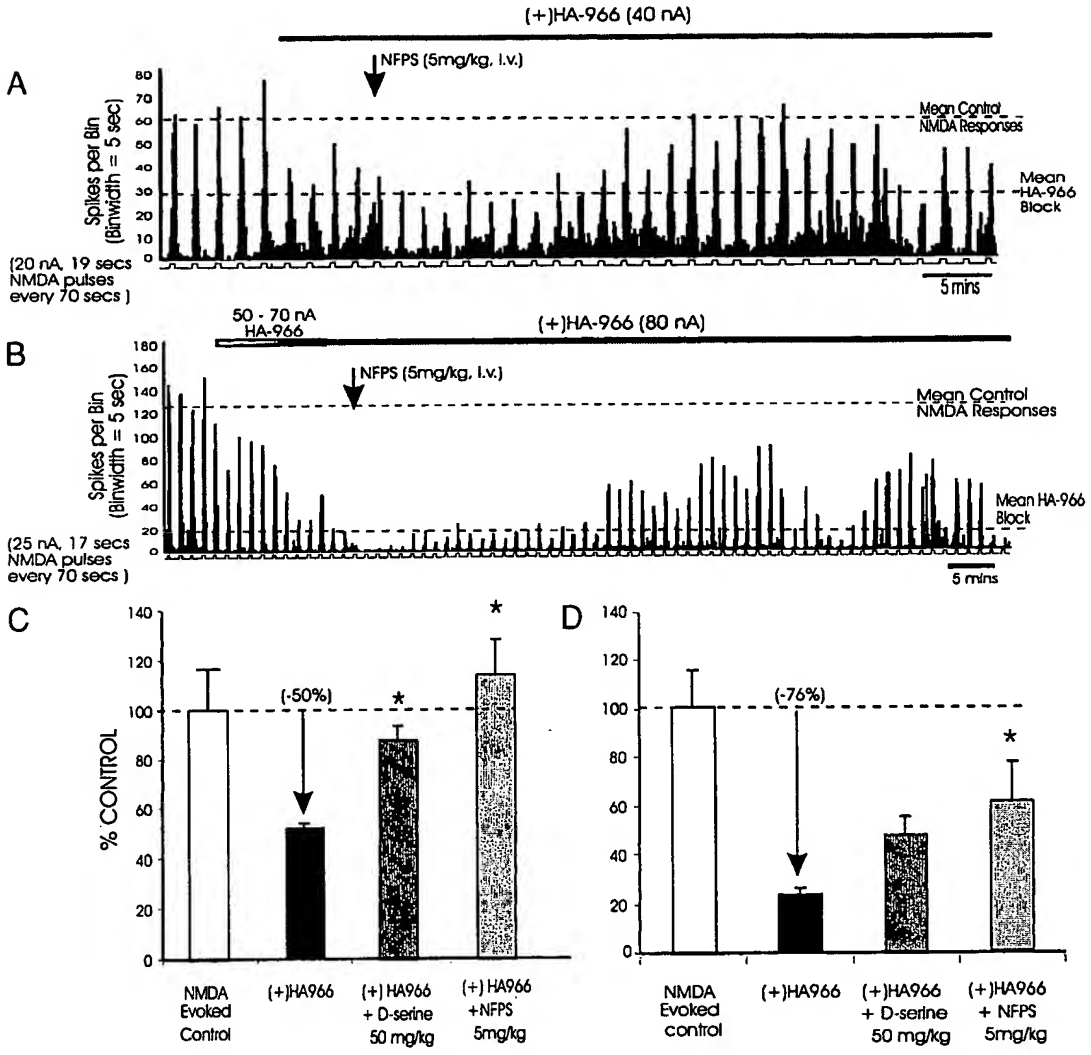

FIG. 6. Both NFPS and D-serine reversed a partial blockade of NMDA-evoked firing by continuous GlyB site antagonist (+)HA-966. *A*: frequency-time histogram illustrating that a partial blockade (≈ −50%) of the NMDA-evoked firing by continuous iontophoretic application of the GlyB site antagonist (+)HA-966 (+40 nA) was completely reversed back to control responses following blockade of the GlyT1 by NFPS (5 mg/kg iv). *B*: frequency-time histogram illustrating that following a more substantial blockade of the NMDA-evoked firing (≈ −90%) by gradual increment of continuous iontophoretic application of (+)HA966 (50–80 nA), intravenous NFPS (5 mg/kg) could only reverse the NMDA-evoked responses partly back to control. *C*: group data histograms summarizing the complete reversal of the partial (+)HA966 block (≈50%) of the NMDA-evoked firing responses by D-serine and NFPS ($n = 4$). *$P < 0.05$ compared with (+)HA966 group responses. *D*: group data histograms summarizing only a partial reversal of a greater (+)HA966 block (>80%) of the NMDA-evoked firing responses D-serine and NFPS. *$P < 0.05$ compared with (+)HA-966 group responses ($n = 4$).

these results suggest that the GlyB site on the NMDA receptor in PFC are likely to be unsaturated in vivo and can be modulated by manipulating extracellular glycine levels near the NMDA-R at the glutamate synapses.

In nominal absence of extracellular glycine [high-performance liquid chromatography (HPLC) analysis of ACSF showed <100 nM glycine present], NMDA EPSC can clearly be synaptically evoked and pharmacologically isolated in our study. Endogenous GlyB site NMDA agonists such as D-serine might be present in the slices and acted as a co-agonist. Since D-serine is not taken up by GlyT1 (Broer et al. 1990; Ribeiro et al. 2002; Schell et al. 1995, 1997; Snyder and Ferris 2000; Snyder and Kim 2000), it can positively modulate evoked NMDA EPSCs in the absence of glycine. When no glycine is included in the perfusate, NFPS potentiated the NMDA EPSCs only at a concentration of 0.01 μM. When a physiological level of extracellular glycine concentration (10 μM) is present in the ACSF, NFPS dose-dependently augments synaptically evoked NMDA EPSC in PFC slices. Further increase in the concentration of NFPS (0.1–1 μM) did not additionally augment the NMDA EPSCs. This suggests that NFPS may only block the uptake of a steady-state level of a trace amount of endogenous glycine present in the slice (<100 nM measured) to augment the NMDA EPSC.

At high doses of glycine (100 μM) or NFPS (10 μM; in the presence of 10 μM glycine), the NMDA EPSC peak was potentiated, but the slow decay time constant did not differ from the control. One possibility is that the enhanced NMDA EPSC will increase enough Ca^{2+} influx via NMDA-R to activate calcineurin to cause a glycine-insensitive NMDA-R desensitization (Legendre et al. 1993; Rosenmund et al. 1995; Tong et al. 1995). Thus at a higher concentration of extracel-

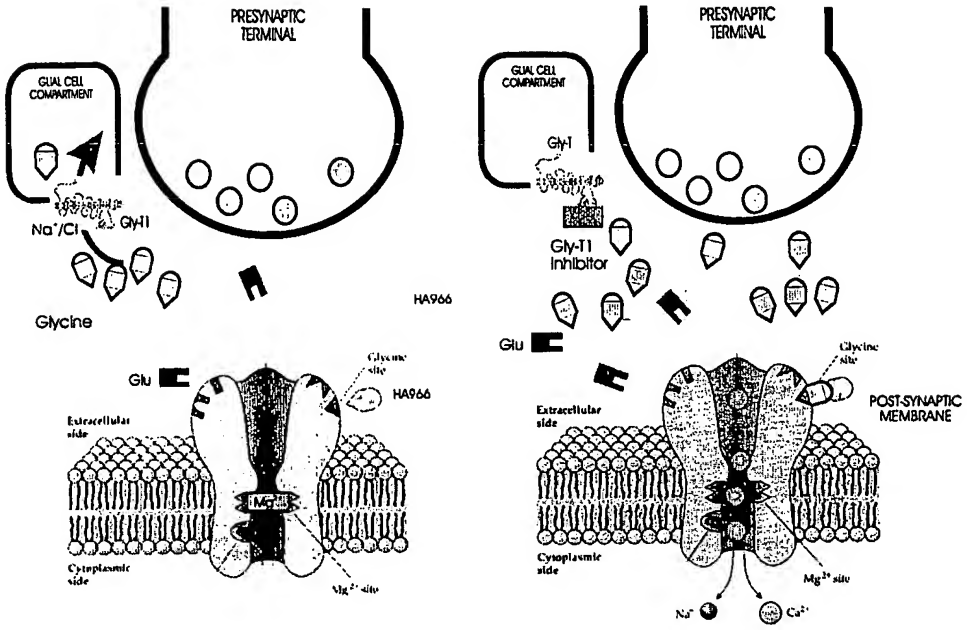

FIG. 7. A schematic model illustrating the proposed mechanisms of GlyT1 action near a NMDA synapse in PFC. *Left*: GlyT1 is located on the glial compartment adjacent to a glutamatergic synapse. Under physiological condition, glycine is actively transported into the glial cell by a Na^+/Cl^--dependent GlyT1. Postsynaptic glutamate activation of *N*-methyl-D-aspartate receptor (NMDA-R) requires co-activation by extracellular glycine for NMDA-R channel opening. *Right*: when the GlyT1 is blocked, glycine transport into the glial cell is impaired. Under this condition, extracellular glycine level near the NMDA-R is elevated. Under this condition when synaptic glutamate release occurs, more glycine is readily available for co-activation of the NMDA-R and potentiates NMDA-R function.

lular glycine, the secondary events (i.e., onset of Ca^{2+}-mediated NMDA-R desensitization) can prevent this glycine prolongation of the late τ_{Slow}, while the high glycine continued to increase early NMDA receptor channel openings (see Fig. 2 and Table 1 in Parsons et al. 1993).

The issue regarding a glycine saturation of the high-affinity GlyB (K_i = 0.1–0.3 μM) site of the NMDA-R in vivo has been controversial (see INTRODUCTION). Although microdialysis studies in freely moving animals have shown that the extracellular concentration of glycine in the rat PFC may be as high as approximately 10 μM (Hashimoto et al. 1995), glycine levels at the GlyB site of the NMDA-R at the synapse may be much less than 1 μM due to the efficient glycine uptake by the GlyT1 (Supplisson and Bergman 1997). Our in vivo electrophysiological recordings showed that by blocking the GlyT1 using NFPS, NMDA-evoked firing of PFC neurons was progressively potentiated. Furthermore, systemic injection of the GlyB site agonist D-serine alone also enhanced the NMDA-evoked firing of single PFC neurons.

Our iontophoretic experiments also showed that the target site of D-serine is likely to be the GlyB site of the NMDA receptor. When the GlyB site antagonist (+) HA-966 was iontophoretically applied continuously to partially block (50 or 80%) the excitatory responses to iontophoretic NMDA applications, intravenous injection of either the GlyB site agonist D-serine or the GlyT1 inhibitor NFPS reversed the blockade of NMDA-evoked firing by (+)HA-966 in single PFC neurons. D-Serine may directly displace (+)HA-966 from their GlyB site occupancy and stimulate the NMDA GlyB site. D-Serine is known to penetrate the blood-brain barrier moderately better than glycine (Oldendoff 1973). In our present study, intravenous administration of D-serine enabled co-activation of the GlyB site when exogenous NMDA was iontophoretically applied, thus functionally potentiating NMDA-evoked firing and reversed GlyB antagonist (+)HA-966 partial blockade of the NMDA-evoked responses in vivo. On the other hand, NFPS may block GlyT1, resulting in elevation of sufficient endogenous glycine levels near the NMDA receptor to displace (+)HA-966 from the NMDA GlyB site, thus allowing the endogenous glycine to stimulate GlyB site and potentiate NMDA-induced firing. It is likely that the transport actions of GlyT1 and/or intracellular glycine sequestration may exceed the K_d of the glycine-binding site and help to rapidly keep $[glycine]_o$ near the NMDA-R to low levels (e.g., <1 μM) (Supplisson and Bergman 1997). Taken together, this evidence suggests that the GlyB sites on NMDA receptor are not likely to be saturated by endogenous glycine in vitro and in vivo in the PFC.

Although it is used as an exogenous GlyB site agonist in this study, endogenous D-serine is also synthesized in glia cells and is highly concentrated in forebrain areas enriched in NMDA-R (Schell et al. 1995, 1997). D-Serine is not a substrate for the GlyT1, but is transported by the ASCT2 system of transporters (Broer et al. 1990; Ribeiro et al. 2002). Synaptically released glutamate may activate ionotropic glutamate receptors on astroglia to release D-serine. In turn, the released endogenous D-serine can serve to co-activate NMDA-R on adjacent postsynaptic neurons (Baranano et al. 2001; Snyder and Kim 2000). However, during repeated iontophoretic NMDA application to establish a stable baseline prior to any systemic drug injections, we did not observe a gradual increase in NMDA-evoked firing over time. Hence, it is unlikely that the iontophoretic application of exogenous NMDA stimulated glia D-serine release in vivo.

Hypofunction of the glutamate/NMDA receptor system has been implicated in the pathophysiology of schizophrenia (Javitt and Zurkin 1991; Tsai and Coyle 2002). The clinical finding that GlyB site stimulation following administration of a large quantity of exogenous glycine (because of its poor CNS penetration) or D-serine as an adjunct to atypical antipsychotics improves schizophrenic symptoms supports the hypothesis that hypo-NMDA system is likely to be involved in the complex pathophysiology of schizophrenia (Javitt et al. 1994; Tsai et al. 1998). The current finding that blocking the GlyT1 may augment endogenous glycine to a level sufficient to potentiate NMDA-R function in vivo may provide a good rationale to

implement this kind of strategy for treating schizophrenia. Perhaps the more important question remaining is to determine to what extent that potentiation of NMDA-R function is beneficial and to address possible excitotoxicity. Animal studies show a lack of neurotoxic damage following long-term pharmacological glycine exposure (Patel et al. 1990; Shoham et al. 1999) and that other glycine transporter inhibitors reverse behavioral hyperactivity caused by psychotomimetic NMDA receptor blocker ketamine (Javitt et al. 1999). These findings further provide a compelling rationale for using GlyT1 inhibitors to indirectly potentiate NMDA receptor functions safely in schizophrenia.

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Behavioral/Systems/Cognitive

The Glycine Transporter Type 1 Inhibitor *N*-[3-(4′-Fluorophenyl)-3-(4′-Phenylphenoxy)Propyl]Sarcosine Potentiates NMDA Receptor-Mediated Responses *In Vivo* and Produces an Antipsychotic Profile in Rodent Behavior

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Glycine acts as a necessary coagonist for glutamate at the NMDA receptor (NMDAR) complex by binding to the strychnine-insensitive glycine-B binding site on the NR1 subunit. The fact that glycine is normally found in the brain and spinal cord at concentrations that exceed those required to saturate this site has led to the speculation that glycine normally saturates NMDAR-containing synapses *in vivo*. However, additional lines of evidence suggest that synaptic glycine may be efficiently regulated in synaptic areas by the glycine transporter type 1 (GlyT1). The recent description of a potent and selective GlyT1 inhibitor (*N*-[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)propyl]sarcosine [NFPS]) provides a tool for evaluation of the hypothesis that inhibition of GlyT1 may increase synaptic glycine and thereby potentiate NMDAR function *in vivo*. In the present study, we found that (+)-NFPS demonstrated >10-fold greater activity in an *in vitro* functional glycine reuptake assay relative to the racemic compound. *In vivo*, (+/−)-NFPS significantly enhanced long-term potentiation in the hippocampal dentate gyrus induced by high-frequency electrical stimulation of the afferent perforant pathway. Furthermore, (+)-NFPS induced a pattern of c-Fos immunoreactivity comparable with the atypical antipsychotic clozapine and enhanced prepulse inhibition of the acoustic startle response in DBA/2J mice, a strain with low basal levels of prepulse inhibition. Collectively, these data suggest that selective inhibition of GlyT1 can enhance NMDAR-sensitive activity *in vivo* and also support the idea that GlyT1 may represent a novel target for developing therapeutics to treat disorders associated with NMDAR hypofunction.

Key words: glycine; schizophrenia; c-Fos; prepulse inhibition; long-term potentiation; DBA/2J mouse; NMDA

Introduction

Glycine acts as a necessary coagonist at the NMDA receptor (NMDAR) (Johnson and Ascher, 1987; Thomson et al., 1989) by binding to a strychnine-insensitive site on the NR1 NMDAR subunit as a necessary component for subsequent glutamate binding (Laube et al., 1997). The affinity of glycine for this binding site varies from 0.1 to 3.0 μM depending on the NR2 subunit makeup of the NMDAR complex (for review, see Danysz and Parsons, 1998). Because glycine is normally found at levels between 7 and 10 μM in the CSF (Ferraro and Hare, 1985), some investigators have suggested that glycine normally saturates NMDAR-associated synaptic regions. Recent evidence, however, indicates that glycine may normally exist at nonsaturating concentrations within these areas. Thus, a high-affinity glycine transporter type 1 (GlyT1) has been described with a distribution pattern that closely overlaps NMDAR localization (Smith et al., 1992). Additional support comes from studies in brainstem slices in which glycine potentiated NMDAR function, but only at concentrations in excess of 100 μM (Berger et al., 1998). Collectively, these results suggest that GlyT1 may tightly control glycine modulation of NMDAR function in the synaptic region. On the basis of these and similar studies, it has been proposed that inhibitors of GlyT1 may be useful for disease states associated with NMDAR hypofunction (e.g., schizophrenia). Inhibitors of GlyT1 are predicted to promote NMDAR function independent of toxic effects expected after administration of direct-acting NMDAR agonists. However, inhibitors of GlyT1 could only be effective if glycine does not saturate relevant NMDAR-associated glycine binding sites *in vivo*. Although slice studies (Berger et al., 1998) are encouraging, the relevance of such findings to NMDAR-dependent activity *in vivo* remains unclear. *N*-[3-(4′-Fluorophenyl)-3-(4′-phenylphenoxy)propyl]sarcosine (NFPS) has been described recently as a potent and selective human GlyT1 inhibitor (Atkinson et al., 2001; Aubrey and Vandenberg, 2001). Using whole-cell patch-clamp recordings from hippocampal pyramidal neurons, Bergeron et al. (1998) demonstrated that NFPS enhanced NMDAR-mediated current in the presence of glycine levels approximating those found *in vivo*. Using *in vivo* microdialysis, Atkinson et al. (2001) demonstrated that (*R*)-NFPS (ALX 5407)

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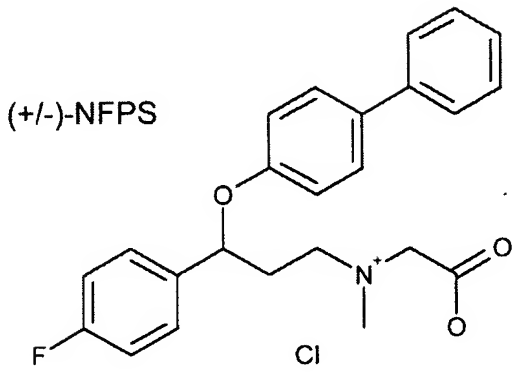


Figure 1. Chemical structure of NFPS (*N*-[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)propyl] sarcosine).

produced a modest, albeit significant, enhancement of extracellular glycine levels in the prefrontal cortex, suggesting that even greater increases in glycine may have occurred in the synaptic region. These findings, however, do not directly address the question of whether the glycine site on the NMDAR is saturated, or the role of the glycine transporter in regulating NMDAR function in glutamatergic synapses *in vivo*.

Accordingly, the present studies examined the role of NFPS administration on *in vivo* functional activity with known sensitivity to manipulation of NMDAR systems. An initial aim of this work was to characterize racemic NFPS and its component enantiomers *in vitro*. A second aim was to characterize the effect of NFPS administration *in vivo*. Specifically, we evaluated the effect of NFPS administration on the following: (1) regional expression of the immediate early gene c-Fos, (2) *in vivo* long-term potentiation (LTP), and (3) prepulse inhibition of the acoustic startle response (PPI) in a DBA/2J mouse strain.

Materials and Methods

In vitro *uptake assay*

Materials. [^{14}C]Glycine (112.7 mCi/mmol) was obtained from PerkinElmer Life Sciences (Emeryville, CA). All of the chemicals were purchased from Sigma (St. Louis, MO).

Compound synthesis. (+/−)-NFPS [(*R*,*S*)-NFPS] was synthesized at Merck Research Laboratories as a racemic mixture (chemical structure is depicted in Fig. 1). This mixture was resolved by chiral HPLC into its component enantiomers, (+)-NFPS and (−)-NFPS, respectively.

Uptake measurement. For uptake experiments, HEK-293 cells expressing rat GlyT1a or rat GlyT2 were cultured in 96-well scintillating Cytostar-T microplates (Amersham Biosciences, Arlington Heights, IL) (Mallorga et al., 2003). Culture medium was removed from the Cytostar plate, and cells were incubated with 30 μl of TB1A buffer (120 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM L-alanine, pH 7.5) with or without drug. Then, 30 μl of [^{14}C]glycine diluted in TB1A was added to each well to give a final concentration of 10 μM unless otherwise specified. After incubation at room temperature for 3 hr, sealed 96-well Cytostar plates were counted on a Top Count (Packard, Meridian, CT). Nonspecific uptake of [^{14}C]glycine was determined in the presence of 10 mM cold glycine. Uptake data represent the mean of at least triplicate determinations. Data were analyzed by nonlinear regression analysis using Prism software (Graph Pad, San Diego, CA).

c-Fos expression assay

Animals. Male Sprague Dawley rats (200–250 gm; Taconic, Germantown, NY) were housed in pairs with access to food and water *ad libitum*. Rats were acclimated to frequent handling before study onset to reduce handling stress to the animals during the experimental protocol. Intraperitoneal injections were performed with the following treatments: (+)-NFPS (10 mg/kg) in 20% β-cyclodextrine, pH 6–7, clozapine (20 mg/kg) in 2% lactic acid, pH 5, and control (20% β-cyclodextrine, pH 6–7). Two hours after the injection, animals were euthanized by CO_2 inhalation and perfused with saline (250 ml) followed by cold, freshly prepared paraformaldehyde (4%). Brains were removed, postfixed in perfusion solution, and cryoprotected with 30–40% sucrose in phosphate buffer (0.1 M).

Immunocytochemistry. Coronal sections (40 μM thick) were cut from each region of interest on a freezing microtome and collected in PBS. Sections were incubated in 10% normal donkey serum (Jackson ImmunoResearch, West Grove, PA) for 10 min, and subsequently washed with anti-c-Fos rabbit antibody (~1 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA) diluted in PBS containing 0.1% Triton X-100 overnight at 4°C. Sections were rinsed with PBS and incubated with biotin-conjugated donkey anti-rabbit antibody (1/1000; Jackson ImmunoResearch) containing 1% normal donkey serum. Bound antibodies were detected using streptavidin conjugate Vector Elite ABC kit (Vector Laboratories, Burlingame, CA), and signal was visualized with diaminobenzidine (Sigma). Sections were dried, mounted on slides, and prepared for observation by microscope.

Counting of positive cells. Quantification of c-Fos-positive cells was performed in the prefrontal cortex, nucleus accumbens, and two regions of the striatum as reported previously (Robertson et al., 1994; Wan et al., 1995). The number of c-Fos-positive cells was computed within a 500 μm^2 surface area in each region. For each rat studied, c-Fos cells were counted in six consecutive sections of each brain region. A one-way ANOVA was performed, and if significant ($p < 0.05$), a Newman–Keuls multiple comparison test was carried out.

In vivo *long-term potentiation*

Animals. Male Sprague Dawley rats (Taconic) were used. All of the animals were allowed access to food and water *ad libitum* before testing. Animals were housed and tested in an Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited facility in strict compliance with all of the applicable regulations.

Procedure. Rats were anesthetized with 1.2–1.5 gm/kg urethane intraperitoneally (Sigma). Under urethane anesthesia, a polyethylene catheter was inserted into the jugular vein of the rats for the subsequent delivery of NFPS or vehicle (50% polyethylene glycol–20% polypropylene glycol–30% water). Rats were placed in a stereotaxic frame, and the skull was exposed. Using a steel burr and microdrill, small holes were stereotaxically placed over the site of the hippocampal dentate gyrus (anterioposterior, −4.0; lateral, +2.0; horizontal, −3.5) and the ipsilateral perforant path (anterioposterior, −7.5; lateral, +4.0; horizontal, −3.3) according to the atlas of Paxinos and Watson (1998). Electrical stimulation was delivered to the perforant path via a bipolar stimulating electrode (Rhodes Electrodes, Woodland Hills, CA) and recorded on a bipolar electrode constructed from Teflon-insulated stainless steel (A-M Systems, Carlsborg, WA). EPSP–population action potential (pop-spike) responses were evoked via a 0.1 msec electrical pulse delivered at a rate of 0.05 Hz using a Grass (West Warwick, RI) S88 stimulator and SIU5 stimulus isolation unit. Before the initiation of each experiment, an input–output relationship was established by increasing the voltage in a stepwise manner until the maximum EPSP response was obtained. The voltage required to produce ~60% of the maximal EPSP slope was used for the remainder of the experiment. After a 30 min baseline period, the rat was injected with the vehicle or (+/−)-NFPS at a volume of 1 cc/kg. Test compound injections were infused by injection pump (Harvard Apparatus, Holliston, MA) at a rate of 0.05 ml/min. Immediately after the injection, the catheter was flushed with 0.5 cc of vehicle to ensure complete delivery of the targeted dose. A high-frequency tetanus (five trains; 80 msec in duration; 20 pulses/train; 0.1 msec/pulse; three times baseline stimulation voltage) was delivered 30 min after test compound administration to induce long-term potentiation. Recording continued for an additional 2 hr post-tetanus. EPSP slope was the primary measure used to evaluate the effect of drug treatment. Data were analyzed using repeated measures ANOVA. An effect was considered significant when $p \leq 0.05$.

Prepulse inhibition

Animals. Male DBA/2J (6–8 weeks of age; The Jackson Laboratory, Bar Harbor, ME), 129S6/SvEvTac, and C57BL/6 (5–10 weeks of age; Tac-

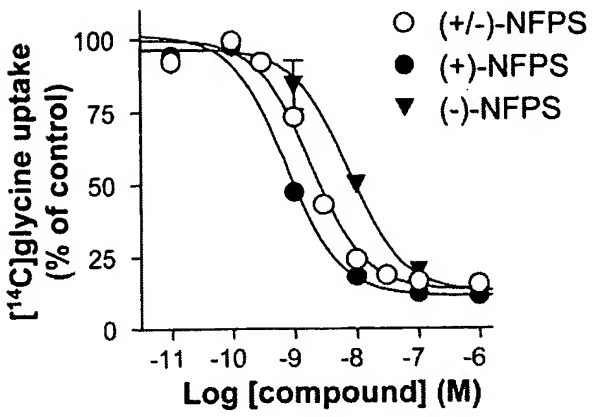
onic) mice were used in the present studies. All of the mice were allowed access to food and water *ad libitum* before testing. Mice were housed on a reverse dark/light cycle (lights off at 6:00 A.M.) and tested in an AAALAC-accredited facility in strict compliance with all of the applicable regulations.

Procedure. SR-Lab (San Diego Instruments, San Diego, CA) acoustic startle chambers were used in the present studies. SR-Lab software controlled the delivery of all of the stimuli to the animals and recorded the response. Startle amplitude was measured as the mean value during a 65 msec period beginning at the onset of the startle-eliciting stimulus. Before the first session of any day, the chambers were calibrated for both movement, using equipment provided by SR-Lab, and for sound levels, using a Tandy sound level meter. In each session, animals were randomly assigned to an experimental group, received (+)-NFPS or vehicle (25% 2-hydroxypropyl-β-cyclodextrin–75% water, pH adjusted to ~6 using 1N NaOH) by intraperitoneal injection, and were placed in the chambers 120 min postinjection. Clozapine-treated mice received 6 mg/kg clozapine intraperitoneally 20 min before placement in the testing chambers. The choice of a 20 min pretreatment time for clozapine was derived from previously reported studies using a similar paradigm (Olivier et al., 2001), whereas a 120 min pretreatment time was selected for NFPS because of the irreversible nature of binding to the GlyT1 site (Atkinson et al., 2001). Animals were given a 5 min acclimation period during which a 65 dB background noise was continuously present. This background noise remained present throughout the entire testing session. After the acclimation period, animals received a series of five 40 msec 118–120 dB bursts of white noise to partially habituate the animals to the startle-eliciting stimulus (Davis, 1988). After these five presentations, the test session, which consisted of 10 repetitions of trials, began. Six different trial types were presented during the session. These consisted of the following: a 10 msec prepulse at 70, 75, 80, or 85 dB (i.e., 5, 10, 15, and 20 dB above background noise) followed 100 msec later by the 118–120 dB 40 msec startle pulse (prepulse pulse conditions), the startle pulse alone (pulse alone), and a period during which no stimulus was presented. Previous studies in our laboratory had determined that these prepulse intensities were insufficient to induce a significant startle response independent of the startle stimulus. The stimuli were presented in random order with interstimulus intervals averaging 15 sec. Levels of prepulse inhibition were determined by the following formula: 100 − [(prepulse pulse/pulse alone) × 100], and expressed as the percentage of prepulse inhibition ± SEM. Data were analyzed using repeated-measures ANOVA with the prepulse intensity as the within-group factor followed by analyses of simple main effects and, when appropriate, *post hoc* analysis using the Dunnett procedure. An effect was considered statistically significant when $p \leq 0.05$.

Results

In vitro characterization

Consistent with a previous report (Atkinson et al., 2001), glycine uptake experiments performed on HEK-293 cells expressing recombinant rat GlyT1a and GlyT2 showed that (+/−)-NFPS is a potent and selective GlyT1a inhibitor. We extended this previous finding by separating the enantiomers of racemic NFPS to determine the activity of each enantiomer on glycine uptake. Interestingly, pharmacological analysis revealed that (+)-NFPS is the most potent enantiomer with a 0.7 nM IC_{50} and high selectivity for rGlyT1a versus rGlyT2 (Fig. 2). Both (+)-NFPS and (+/−)-NFPS were used to perform the experiments reported below.



	rGlyT1a	rGlyT2
(+/-)-NFPS	9.8 ± 0.1 nM	>50 μM
(+)-NFPS	0.7 ± 0.1 nM	>100 μM
(-)-NFPS	30 ± 11 nM	>100 μM

Figure 2. Competition experiments revealed that (+/−)-NFPS and its enantiomers fully antagonized [^{14}C]glycine (10 μM) uptake in HEK-293 cells recombinantly expressing rat GlyT1a with IC_{50} values shown in Table 1. Similar experiments with rat GlyT2 revealed the subtype selectivity of these compounds. Data are the mean IC_{50} values ± SEMs from three experiments. Nonspecific uptake was determined in the presence of 10 mM cold glycine. Error bars represent SEMs.

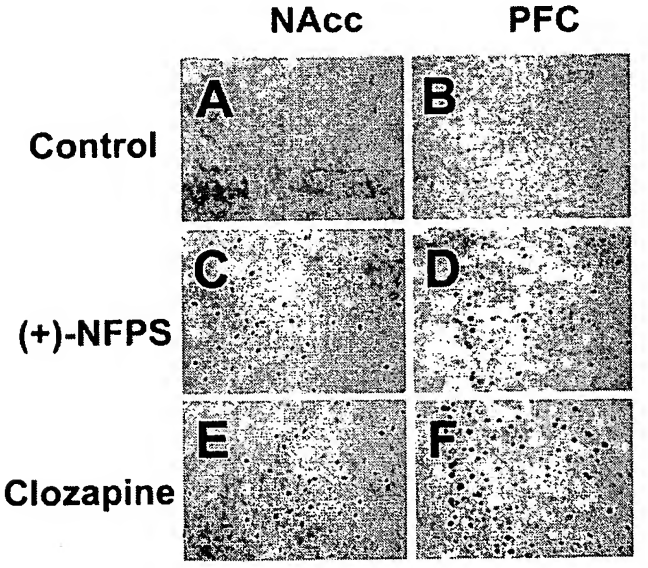


Figure 3. Photomicrographs illustrating the expression of c-Fos immunoreactivity in nucleus accumbens (NAcc) (left column) and prefrontal cortex (PFC) (right column) of rats treated with vehicle (*A*, *B*), (+)-NFPS (10 mg/kg, i.p.) (*C*, *D*), and clozapine (20 mg/kg, i.p.) (*E*, *F*).

c-Fos expression

As illustrated in Figure 3, (+)-NFPS and clozapine produced a marked increase in the number of cells displaying c-Fos immunoreactivity in the nucleus accumbens. Quantitative analysis revealed a significant fourfold to fivefold increase in c-Fos cells 2 hr after (+)-NFPS and clozapine treatment (Table 1). Similar threefold and sixfold increases in c-Fos in the prefrontal cortex were found in rats receiving (+)-NFPS and clozapine, respectively (Fig. 3, Table 1). In the striatum, (+)-NFPS and clozapine produced a similar and specific pattern of c-Fos expression. Neither drug induced a significant increase in c-Fos expression in cells located in the dorsolateral striatum, whereas in the medial portion of this brain structure, a significantly higher number of c-Fos-positive cells was observed after clozapine treatment (Table 1).

Long-term potentiation

As depicted in Figure 4, application of high-frequency tetanic stimulation induced a long-lasting enhancement of dentate gyrus

Table 1. Summary of group data for c-Fos studies

	PFC	NAcc	M Str	DL Str
Control	18 ± 4	19 ± 4	9 ± 1	8 ± 2
(+)-NFPS	52 ± 10*	80 ± 12**	18 ± 5	13 ± 2
Clozapine	112 ± 12***	104 ± 10***	29 ± 2**	14 ± 1

PFC, Prefrontal cortex; NAcc, nucleus accumbens; M Str, medial striatum; DL Str, dorsolateral striatum.

Data are means ± SEMs from 24 sections per area and 12 sections per area for control and (+)-NFPS rats ($n = 4$) and clozapine rats ($n = 3$), respectively.

ANOVA ($p < 0.05$) followed by Newman–Keuls multiple comparison test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus control.

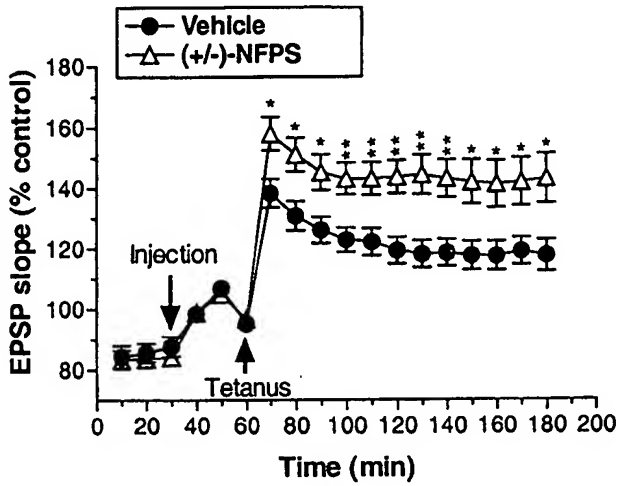


Figure 4. Group data depicting the effect of (+/−)-NFPS (3 mg/kg, i.v.) administration on LTP in the hippocampal dentate gyrus produced by delivery of a high-frequency electrical stimulation of the perforant path in anesthetized rats. An injection of vehicle or (+/−)-NFPS was administered 30 min after the initiation of each experiment and 30 min before the delivery of the tetanic stimulation. After stimulation of the perforant pathway, NFPS-treated rats displayed a significantly greater magnitude of LTP that was maintained for the duration of the testing period. Data are grouped in 10 min bins, and asterisks represent a significant difference from vehicle-treated rats: *$p < 0.05$, **$p < 0.01$. Error bars represent SEMs; $n = 10$ per group.

EPSP slope (LTP) after administration of both vehicle (~20% increase over baseline) and (+/−)-NFPS (~50% increase over baseline). LTP persisted throughout the 2 hr posttetanus data collection period. Treatment with (+/−)-NFPS (3 mg/kg, i.v.) significantly enhanced LTP relative to vehicle treatment as reflected by a significant main effect of treatment ($F_{(1,18)} = 8.81$; $p < 0.009$) and a significant treatment by time interaction ($F_{(17,306)} = 6.1$; $p < 0.004$). Additional analysis comparing only the time points before tetanus revealed a lack of basal difference between treatment groups ($p > 0.61$), suggesting that the overall treatment effects were not attributable to baseline differences between groups. This was further confirmed by the finding of a significant main effect of treatment ($F_{(1,18)} = 9.96$; $p < 0.006$), but no significant treatment by time interaction ($p > 0.56$) when only the data from the time points after the tetanus were included in the analysis.

Prepulse inhibition

An initial study was conducted wherein DBA/2J mice were compared with two additional strains commonly used in the research environment (129S6 and C57BL/6). As depicted in Figure 5, both 129S6 and C57BL/6 mice displayed significantly higher levels of PPI across multiple prepulse intensities relative to DBA/2J mice. This was confirmed by the finding of a significant main effect of mouse strain ($F_{(2,23)} = 27.2$; $p < 0.001$) and a significant strain × prepulse intensity interaction ($F_{(6,69)} = 5.7$; $p < 0.001$).

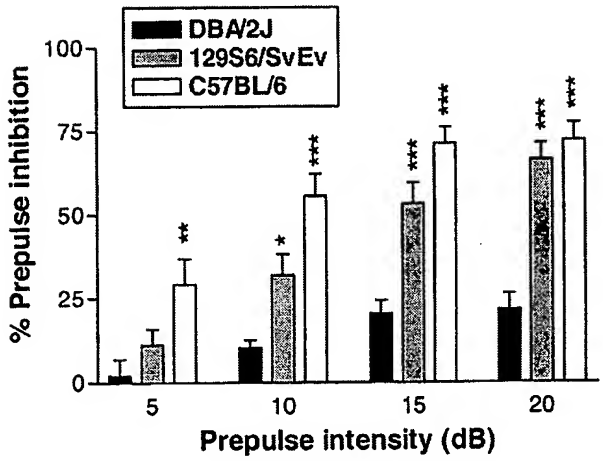


Figure 5. Group data depicting basal PPI in three mouse strains at four prepulse intensities (5–20 dB above background). DBA/2J mice showed significantly lower levels of PPI relative to the 129S6 and C57BL/6 strains. Asterisks represent a significant difference from DBA/2J mice: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Error bars represent SEMs; $n = 8$ per group.

DBA/2J mice were further examined after administration of vehicle, (+)-NFPS (1 and 10 mg/kg, i.p.), and clozapine (6 mg/kg, i.p.). Although not systematically examined in the current studies, NFPS administration was generally well tolerated during the relatively short time frame of these acute studies. Thus, no enhanced mortality or overt adverse behavioral effects were noted. As depicted in Figure 6, both NFPS and clozapine enhanced PPI in this strain of mouse. Interestingly, NFPS (10 mg/kg) and clozapine were effective in enhancing PPI at all of the prepulse intensities examined, whereas the lower dose of NFPS (1 mg/kg) only enhanced PPI at the two highest prepulse intensities examined (i.e., 15 and 20 dB above background). These results were confirmed by the finding of a significant main effect of treatment ($F_{(3,65)} = 11.36$; $p < 0.001$). Furthermore, the lack of a significant treatment × prepulse intensity interaction ($p > 0.09$) suggests that the effects of these treatments enhance PPI in these mice regardless of the prepulse intensity examined. It is noteworthy that the changes in PPI that occurred after NFPS treatment occurred independent of any significant change in basal startle amplitude as assessed by the response of the mice to the pulse-alone condition (Fig. 6*B*). In contrast, clozapine at this dose did significantly impair basal startle amplitude (overall effect, $F_{(3,65)} = 4.9$; $p < 0.005$) (see Fig. 6 for the result of *post hoc* analyses).

Discussion

The results of the present study confirm that NFPS is a potent and selective inhibitor of rat GlyT1a. The results also demonstrate that the administration of this compound *in vivo* produces activity consistent with those expected after potentiation of glycine–NMDAR function and further suggest an antipsychotic and/or procognitive profile of this compound in these preclinical animal studies.

The suggestion that potentiation of NMDAR function may be useful for the treatment of schizophrenia is derived from the corollary observation that NMDAR hypofunction may be critically involved in the etiology or symptoms associated with this disease. Thus, NMDAR antagonists, such as phencyclidine (PCP) and ketamine, induce psychotic states in normal human volunteers and exacerbate existing symptomatology in schizophrenic patients (Olney et al., 1999). Furthermore, increases in NMDAR density has been reported in a variety of brain regions of schizo-

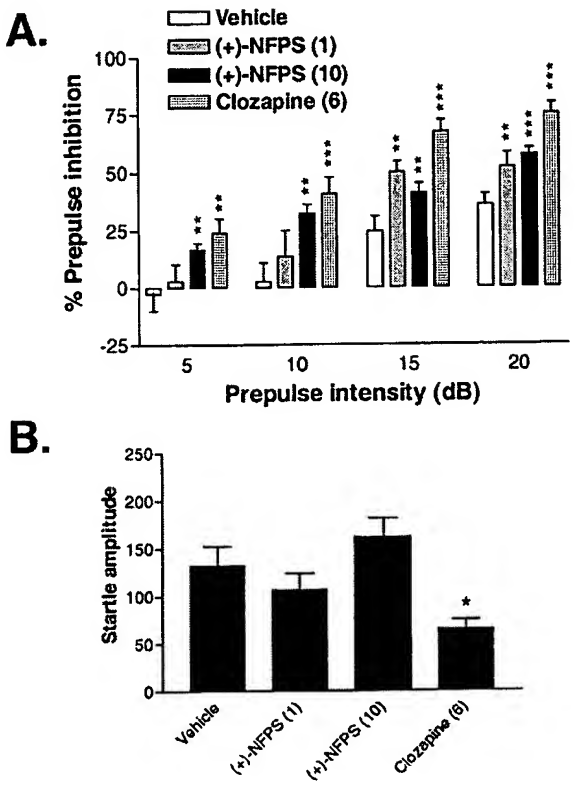


Figure 6. *A*, The effect of vehicle, two doses of NFPS (1 and 10 mg/kg, i.p.), and clozapine (6 mg/kg, i.p.) on PPI in DBA/2J mice at four prepulse intensities (5–20 dB above background). Vehicle and NFPS were administered 120 min before placement in the testing apparatus, whereas clozapine was administered 20 min before testing. Asterisks represent a significant difference from the vehicle group: $^{**}p < 0.01$, $^{***}p < 0.001$. Error bars represent SEMs. *B*, The effect of vehicle, NFPS, and clozapine on startle amplitude during pulse-alone trials in the same mice represented in *A*. The asterisk represents a significant difference from the vehicle group: $^{*}p < 0.05$. Error bars represent SEMs.

phrenic patients (Ishimaru et al., 1992, 1994). In specific regard to a role for glycine in the treatment of schizophrenia, recent genetic evidence suggests that a polymorphism in a primate-specific gene (G72) may be linked to schizophrenia in Canadian and Russian populations (Chumakov et al., 2002). Interestingly, it has been demonstrated that the protein coded by G72 positively modulates D-amino acid oxidase, which in turn metabolizes D-serine (Chumakov et al., 2002). Because D-serine is a known agonist at the NMDAR-associated glycine binding site (for review, see Hashimoto and Oka, 1997), these data raise the possibility of a primary deficiency of the NMDAR-dependent glycine system in the schizophrenic condition. Consistent with this suggestion, administration of agonists at this allosteric glycine binding site results in a significant symptomatic improvement in schizophrenic patients (Javitt et al., 1994; Goff et al., 1995; Heresco-Levy et al., 1996, 1999, 2002; Tsai et al., 1998).

The ability of NFPS to selectively enhance c-Fos immunoreactivity in the nucleus accumbens and prefrontal cortex, but not in the dorsolateral striatum, is similar to results produced by a wide variety of atypical antipsychotic drugs (Robertson and Fibiger, 1992; Robertson et al., 1994) and clozapine in this study. In addition, the atypical index of (+)-NFPS as defined by Robertson et al. (1994) is positive (+56), suggesting that (+)-NFPS might be devoid of extrapyramidal side effects that characterize typical antipsychotic drugs such as haloperidol (Robertson et al., 1994; Deutch et al., 1996). Although not reaching statistical significance, the small activation in the medial striatum by (+)-NFPS paralleled the effect of clozapine and is consistent with activation of the prefrontal cortex (Robertson et al., 1994). The present results demonstrate that NFPS-induced c-Fos expression patterns resemble those of clozapine, suggesting that GlyT1 inhibitors may share an atypical antipsychotic profile. Although both typical and atypical antipsychotic-induced c-Fos expression appears to be dependent on intact NMDA receptor function (Leveque et al., 2000), the cellular and molecular mechanisms involved likely differ between NFPS and typical antipsychotics such as haloperidol. It has been proposed that haloperidol stimulates NMDA receptor activity in the striatum and induces c-Fos expression through disinhibition of protein kinase A and subsequent phosphorylation of a serine residue on the NMDAR NR1 subunit (Leveque et al., 2000). In contrast to this indirect effect on NMDAR function, the stimulating effect of (+)-NFPS on c-Fos expression may result from an increased availability of synaptic glycine and subsequent potentiation of NMDAR synaptic activity. Clozapine may have a direct, albeit mechanistically distinct, action on NMDAR function in that clozapine administration results in a release of excitatory amino acids and enhances electrically evoked EPSPs through a potentiation of NMDA receptors in the rat prefrontal cortex (Daly and Moghaddam, 1993; Arvanov et al., 1997).

It is well established that LTP in the dentate gyrus region of the hippocampus is reliant on activity-dependent NMDAR function *in vivo* (Morris et al., 1986; Abraham and Mason, 1988). Thus, this measure provided a means to assess the impact of increased glycine levels on synaptic NMDAR function. We hypothesized that, if glycine is normally maintained at subsaturating concentrations within these synapses *in vivo*, administration of the GlyT1 inhibitor NFPS should enhance LTP. Consistent with this hypothesis, we showed that NFPS administration resulted in significantly greater enhancement of LTP relative to vehicle treatment. This finding extends previous reports *in vitro*, in which glycine enhanced LTP (Tauck and Ashbeck 1990; Watanabe et al., 1992), to an *in vivo* preparation. Given the postulated role of LTP as a molecular mechanism underlying memory formation (Lynch, 1998), these results further allow for the possibility that selective inhibitors of GlyT1 may enhance learning and memory processes.

Previous published studies (Toth and Lajtha, 1986; Javitt et al., 1997, 1999) examining the behavioral effects of glycine agonists or GlyT inhibitors have been limited to the demonstration that glycine, the glycine uptake inhibitor glycyldodecylamide, and several novel glycineamide derivatives specifically inhibit PCP- but not amphetamine-induced hyperlocomotion in mice. In an effort to extend these findings using NFPS, we examined the role of GlyT1 inhibition on PPI in DBA/2J mice. PPI is a well characterized measure of sensorimotor gating that is deficient in schizophrenic patients and in animals after treatment with NMDAR antagonists (for review, see Geyer et al., 2001). The DBA/2J strain of mice displays low levels of prepulse inhibition relative to alternate strains of mice (Olivier et al., 2001) (see also Results). PPI is significantly enhanced in these mice after treatment with clozapine, risperidone, haloperidol, and raclopride (McCaughran et al., 1997; Olivier et al., 2001), leading to the suggestion that this strain of mouse may provide a model system for the examination of novel antipsychotic drug agents (Olivier et al., 2001). In the present study, we confirmed that DBA/2J mice display low levels of basal PPI relative to 129S6 and C57BL/6 mouse strains. Furthermore, we demonstrated that NFPS enhances PPI in this strain of mouse with a level of efficacy comparable with that of clozapine. Interestingly, the enhancement of PPI after administration

of NFPS was observed independent of any change in baseline startle amplitude.

In summary, the present results confirm that NFPS represents a selective and potent inhibitor of GlyT1. Functional *in vivo* studies using c-Fos immunoreactivity, *in vivo* LTP, and PPI behavioral measures add additional support to the suggestion that enhancement of synaptic glycine via blockade of GlyT1 results in augmentation of NMDAR-sensitive functional activity. Collectively, these data support the suggestion that glycine is normally maintained at subsaturating concentrations synaptically and that inhibition of GlyT1 may provide a novel treatment approach for schizophrenia, psychosis, cognitive dysfunction, and related disorders.

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Reversal of Phencyclidine-Induced Dopaminergic Dysregulation by *N*-Methyl-D-Aspartate Receptor/Glycine-site Agonists

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N-methyl-D-aspartate (NMDA) receptors may play a critical role in the pathophysiology of schizophrenia. In rodents, NMDA receptor antagonists, such as phencyclidine (PCP), induce dopaminergic dysregulation that resembles the pattern observed in schizophrenia. The present study investigates the degree to which concurrent treatment with NMDA modulators, such as glycine and the recently developed glycine transport antagonist *N*[3-(4″-fluorophenyl)-3-(4″-phenylphenoxy)propyl]sarcosine (NFPS) prevents dopaminergic dysregulation observed following chronic (3 months) or subchronic (2 weeks) PCP administration. Both chronic and subchronic treatment with PCP in the absence of glycine or NFPS led to significant potentiation of amphetamine-induced dopamine release in the prefrontal cortex and striatum, similar to that observed in schizophrenia. Treatment with either high-dose glycine or NFPS along with PCP prevented PCP effects. These findings demonstrate effective doses of glycine for use in animal models of schizophrenia, and support recent clinical studies showing the effectiveness of NMDA agonists in the treatment of persistent symptoms of schizophrenia.
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Keywords: glutamate; glycine; NMDA receptor; amphetamine; phencyclidine; schizoprenia

INTRODUCTION

Symptoms of schizophrenia have traditionally been attributed to the hyperactivity of brain dopaminergic systems (eg Davis *et al*, 1991; Moore *et al*, 1999). Over the past decade, however, the phencyclidine (PCP) model of schizophrenia has attained increasing prominence (Coyle, 1996; Javitt *et al*, 1987; Javitt and Zukin, 1990, 1991; Jentsch *et al*, 1999; Krystal *et al*, 1994; Newcomer *et al*, 1999). This model is based upon the observation that PCP and related agents such as ketamine induce schizophrenia-like symptoms by blocking *N*-methyl-D-aspartate (NMDA)-type glutamate receptors, and exacerbate symptoms in remitted patients. Further, agents that potentiate NMDA receptor-mediated neurotransmission significantly reduce persistent negative and cognitive symptoms of schizophrenia (Goff *et al*, 1999; Heresco-Levy *et al*, 1999; Javitt *et al*, 2001, 1994; Shoham *et al*, 2001; Tsai *et al*, 1998). Psychotomimetic effects of PCP are observed during both acute and chronic administration. Further, in early studies, behavioral effects of PCP were found to be most severe in patients with postencephalitic Parkinson's disease, suggesting critical interactions with brain dopaminergic systems (Meyer *et al*, 1959).

A recent finding in schizophrenia research has been the demonstration that patients show increased dopaminergic sensitivity to amphetamine challenge. This finding has been replicated in several cohorts using *in vivo* PET and SPECT imaging (Abi-Dargham *et al*, 1998; Breier *et al*, 1998, 1997; Kegeles *et al*, 1999; Laruelle *et al*, 1998, 1999, 1996, 1995, 1997a, b). Similar abnormalities are observed in humans following ketamine administration (Kegeles *et al*, 2000), and in rodents following acute (Balla *et al*, 2003; Miller and Abercrombie, 1996) or chronic (Balla *et al*, 2001b, 2003) NMDA antagonist administration.

NMDA receptors are modulated by amino acids, including glycine and D-serine, which bind to the glycine modulatory site of the NMDA complex. Both glycine (Javitt *et al*, 1999; Toth and Lajtha, 1986) and D-serine (Contreras, 1990; Nilsson *et al*, 1997) have been shown to reverse the behavioral effects of PCP in rodents following acute administration, indicating the *in vivo* relevance of the interaction. The effects of these agents during long-term administration, however, have not been evaluated. The use of D-serine in rodents is contraindicated because of nephrotoxicity (Carone and Ganote, 1975). In contrast, glycine is well tolerated in rodents for up to 5 months at

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doses that produce serum levels similar to those observed during clinical trials in schizophrenia (Shoham *et al*, 2001). The present study, therefore, evaluates the effects of long-term glycine treatment on neurochemical alterations induced by chronic PCP administration.

Occupancy of the glycine site is governed by a glycine (GlyT1) transporter that maintains low, subsaturating glycine levels in the immediate vicinity of NMDA receptors. Recently developed glycine transport inhibitors, such as glycyldodecylamide (GDA) (Javitt *et al*, 1999; Javitt and Frusciante, 1997) or *N*[3-(4″-fluorophenyl)-3-(4″-phenylphenoxy)propyl]sarcosine (NFPS) (Atkinson *et al*, 2001; Herdon *et al*, 2001), increase brain glycine levels *in vivo* (Atkinson *et al*, 2001) and potentiate NMDA receptor-mediated neurotransmission *in vitro* (Bergeron *et al*, 1998). As yet, however, few animal models have been published that show sensitivity to effects of these agents. The present study evaluates the degree to which PCP-induced enhancement of amphetamine-induced DA release in brain may be used to detect the effects of indirect, as well as direct, agonists of the NMDA-associated glycine-binding site.

MATERIALS AND METHODS

Animals

Studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. Male Sprague–Dawley rats (150–200 g) were bred in-house. Animals were maintained under a 10/14 h dark/light cycle, and were allowed food and water *ad libitum* during the microdialysis procedure and during the night-time locomotor activity measurements. Food was withdrawn during amphetamine challenge procedures. Three to nine animals were used per group.

PCP Administration

PCP hydrochloride (obtained from the National Institute of Drug Abuse) was dissolved in sterile physiological saline and administered via an osmotic pump (ALZA Corporation, model 2ML4) implanted under the skin. For 3-month administration, minipumps were replaced monthly to maintain appropriate serum levels. Saline-filled pumps were used in control animals. The pumps were filled based on the animal weight at the start of the experiment to deliver indicated PCP doses. The implantation was carried out under anesthesia with ketamine hydrochloride and acepromazine maleate 1 : 1 mixture (1 µl/g i.m.).

Microdialysis

Microdialysis was performed following either 14 days or 3 months of PCP administration, as indicated. Animals were still receiving PCP at the time of microdialysis. Animals were anesthetized with chloral hydrate (400 mg/kg i.p.) and mounted in a stereotaxic frame (David Kopf Instrument). A CMA 10 guide cannula (Carnegie Medicine) was implanted into the left dorsomedial striatum and/or right prefrontal cortex. The implantation coordinates for the striatum (AP: +1.00, L: 2.5, V: 4.00) and prefrontal cortex PFC (AP: +4.1, L: +1.0, V: −1.2, 20% angle) were determined relative to bregma (Paxinos and Watson, 1998). Cannulae were cemented to the skull using dental acrylic with embedded stainless-steel bone screws.

During the 48 h following surgery, CMA 10 probes (0.5 mm × 2.0 mm or 4.0 mm membrane length with a molecular cutoff 20 000 Da) were inserted into the guide cannulae. The estimated recovery rate was 18–20%. Probes were continuously perfused using a syringe pump CMA/100 (Carnegie Medicine) at a flow rate of 1.0 µl/min with an Mg^{2+}-free Ringer solution containing NaCl 147 mM; KCl 4 mM; $CaCl_2$ 1.2 mM (degassed). A period of 2 h was allowed to establish the basal level of the extracellular catecholamines. Then 30-min dialysate samples were collected with a fraction collector (Bioanalytical Systems). After three baseline samples, the rats were challenged with amphetamine sulfate (RBI), which was dissolved in physiological saline and given subcutaneously at a dose of 1 mg/kg.

Following completion of the experiment, animals were anesthetized with ketamine hydrochloride and acepromazine maleate 1 : 1 mixture (1 µl/g i.m.). Blood samples were obtained via cardiac puncture, and plasma separated for PCP analysis. The rat brain was fixed first with 100 ml of 0.9% saline in 0.1 M phosphate, pH = 7.4, and then with 300 ml of ice-cold 4% paraformaldehyde in 0.1 M phosphate, pH = 7.4. The brains were cryoprotected in 30% sucrose in 0.1 M phosphate. The placement of the probes was determined histologically.

Dopamine Level Determinations

Dopamine levels were determined by high-pressure liquid chromatography with electrochemical detection (HPLC-EC) (BAS-480 system). The dialysate samples (30 µl)—collected in 0.1 N perchloric acid—were injected by autosampler (BAS Sample Sentinel) onto a microbore C_{18} 100 × 2 mm^2 column. The sample was eluted with filtered, degassed mobile phase (NaH_2PO_4 25 mM; sodium citrate 50 mM; disodium-EDTA 27 µM; diethylamine-HCl 10 mM; 1-octanesulfonic acid, sodium salt; methanol 3% v/v; dimethylacetamide 2.1% v/v; pH = 3.5) at a flow rate of 0.4 ml/min, yielding a retention time of 6.0 min. Classic glassy carbon electrodes (BAS) *vs* the Ag/AgCl reference electrode at 0.60 V and sensitivity levels of 0.5 nA were used for dopamine detection.

Data were acquired on an IBM-compatible PC using BAS-5 interface. Standard curves were constructed using 7 pts between 0.625 and 80 pg/10 µl for dopamine. Correlation coefficients (r) of >0.98 were obtained for all curves. The working standard solutions were stored at −80°C and 10 µl of the standard solution was injected between biological samples.

Data Analysis

Primary dependent measures consisted of dopamine levels prior to and following amphetamine administration. Data were analyzed using repeated measures ANOVA with Geisser-Greenhouse correction, with within-subject measure of time following amphetamine injection (ie fraction or observation number) and between-subject factor of drug (PCP or saline). Significant main or interaction effects were

followed up by a between-group *post hoc* *t*-test. Two-tailed statistics with an α level of significance of $p<0.05$ were used throughout. Data in the text are mean $\pm$ SEM.

RESULTS

3-Month Treatment Study

An initial experiment evaluated the effects of glycine on PCP-induced dopaminergic hyper-reactivity during long-term PCP administration, to mimic the likely effects of prolonged clinical treatment with glycine in humans. This was considered the longest time over which rats could technically be maintained on PCP using osmotic mini-pumps. Rats were treated for 3 months with PCP (5 mg/kg/day) or saline with or without concurrent glycine administration. Glycine was administered orally via an enriched diet (8% glycine by weight). Serum PCP levels obtained at the time of killing, 69.3 $\pm$ 7.0 ng/ml, were within the range associated with PCP psychosis in humans (Javitt and Zukin, 1991) and were not significantly different between glycine-treated and control animals ($p>0.2$).

Glycine treatment led to a significant, two-fold increase in serum levels of glycine from 221 $\pm$ 21 to 480 $\pm$ 97 μM ($t=2.73$, $p<0.02$) and a 1.5-fold increase in serine levels from 167 $\pm$ 10 to 238 $\pm$ 21 μM ($t=3.25$, $p<0.01$). Although significantly elevated *vs* control, these levels are somewhat lower than those achieved during clinical treatment with glycine (Heresco-Levy *et al*, 1999; Javitt *et al*, 2001; Leiderman *et al*, 1996). In order to verify that increased serum levels were associated with increased brain concentrations, amino acid levels were determined from pream-phetamine microdialysate samples as well. As in serum, microdialysate glycine levels were increased approximately two-fold in glycine-treated *vs* control animals ($p<0.05$). A 1.4-fold increase was observed for microdialysate serine levels, but the degree of between-group difference did not reach statistical significance (Figure 1). Glutamate, aspartate, and glutamine levels were unchanged.

A 3-month treatment with PCP led to a significant 1.5-fold increase in amphetamine-induced PFC dopamine release *vs* control animals (Figure 2), as reflected in a significant group (PCP/control) $\times$ time interaction during the 0–210 min post-amphetamine treatment interval ($F_{9,54}=4.4$, $p=0.006$). In contrast, in animals receiving a glycine-enriched (8% by weight) diet, no PCP $\times$ time interaction was observed ($F_{9,45}=0.1$, $p=0.9$), although the main and interactive effects of glycine were not significant.

In control animals, the effects were most pronounced 150–210 min following amphetamine administration. A *post hoc* analysis was therefore conducted over this period alone. Over this time period, significant glycine $\times$ PCP ($F_{1,12}=5.2$, $p=0.04$) and glycine $\times$ PCP $\times$ time ($F_{2,24}=4.7$, $p=0.02$) interactions were observed.

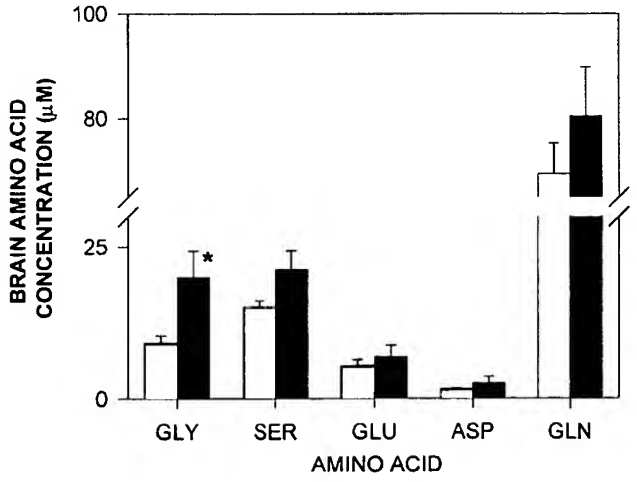

Figure 1 Microdialysis glycine levels in animals receiving regular (open bars, $n=11$) or a high-glycine (8% by weight) diet (solid bars, $n=13$). *$p<0.05$ glycine *vs* control.

2-Week, 16% Glycine Diet

As the glycine levels obtained during the 3-month study were somewhat below those obtained during clinical trials, a second study investigated the effects of a higher glycine dose (16% by diet) for 2 weeks. Owing to the shorter treatment duration, a higher dose of PCP was used (15 mg/kg/day) for this study. The mean PCP levels among treated animals were 85.4 $\pm$ 6.6 ng/ml, with no significant difference between animals receiving regular *vs* high-glycine diet. Serum glycine levels in rats receiving 16% glycine diet, 1236.0 $\pm$ 253.4 nmol/ml, were increased five-fold over levels in rats receiving a regular diet, (238.4 $\pm$ 50.7 nmol/ml, $t=3.93$, $p<0.001$) and within the range obtained during clinical studies with glycine (Heresco-Levy *et al*, 1999; Javitt *et al*, 2001; Leiderman *et al*, 1996).

For this study, dopamine levels were measured in both the frontal cortex and striatum (Figure 3). In animals receiving a regular diet (left panels), subchronic PCP treatment led to a significant increase in amphetamine-stimulated DA release in both the PFC and striatum, as reflected in the significant PCP $\times$ time effects (FC: $F_{9,252}=6.9$, $p<0.001$; STR: $F_{9,153}=5.8$, $p<0.001$). These effects were prevented in animals receiving a high-glycine (16% by weight) diet, as reflected in the absence of significant PCP $\times$ time effects ($F_{9,153}=1.3$, $p>0.2$; STR: $F_{9,126}=0.2$, $p>0.9$).

In this data set, PCP treatment led to significant potentiation of amphetamine-stimulated dopamine release throughout the 30–210 min period in both the PFC ($F_{1,43}=13.5$, $p=0.001$) and striatum ($F_{1,33}=4.66$, $p<0.04$). This effect was reversed by glycine treatment as indicated by a significant glycine $\times$ PCP interaction in both

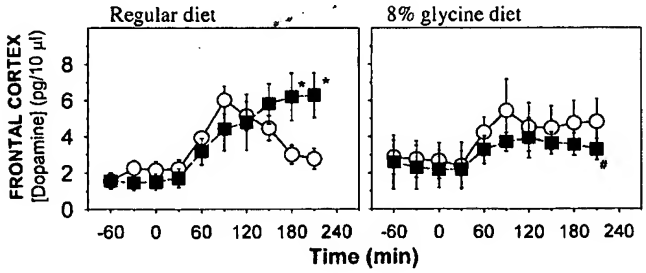

Figure 2 Effect of GLY on AMPH-induced DA release in PFC in animals treated with 5 mg/kg/day PCP for 12 weeks (filled squares) or saline-treated controls (open circles). $n=4$–6 per group. *$p<0.05$ PCP *vs* control, #$p<0.05$ PCP + high-glycine *vs* PCP + regular diet.

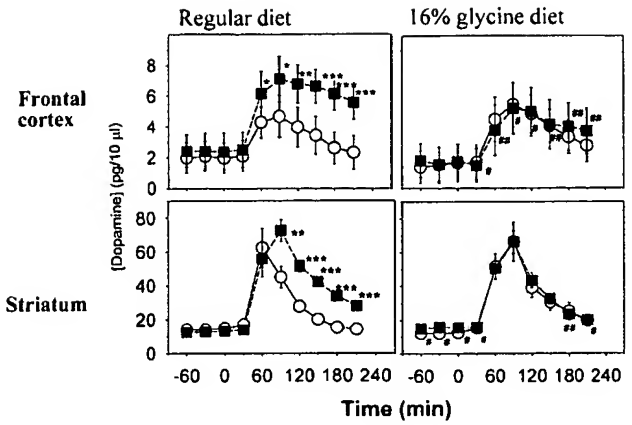

Figure 3 Effect of glycine on amphetamine-induced DA release in PFC (top) and striatum (bottom) in animals treated with 15 mg/kg/day PCP for 2 weeks (filled circles) or saline-treated controls (open circles). $n = 7$–20 per group. $^{*}p < 0.05$ *vs* control, $^{**}p < 0.01$, $^{***}p < 0.001$; $\#p < 0.05$ PCP + high-glycine *vs* PCP + regular diet, $\#\#p < 0.01$.

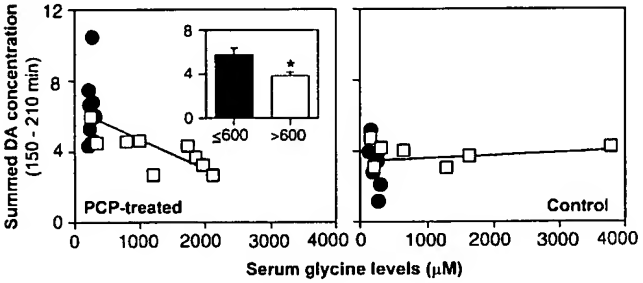

Figure 4 Relationship between serum glycine levels and PFC DA levels during the 150–210 min post-amphetamine-treatment time period in animals receiving PCP (left) and controls (right). Filled symbols indicate animals receiving a regular diet. Open symbols indicate animals receiving a high-glycine diet. Inset Bar chart showing the mean (SEM) PFC DA levels during the 150–210 min period for PCP-treated animals with serum glycine levels ≤ 600 µM (filled bar, $n = 12$) *vs* animals with the serum glycine levels > 600 µM (open bar, $n = 6$). Regardless of diet, animals with serum glycine levels ≤ 600 µM showed significantly lower levels of amphetamine-induced DA release than animals with serum glycine levels > 600 µM. $^{*}t = 2.91$, $p = 0.01$ ≤ 600 µM *vs* > 600 µM.

brain regions (PFC: $F_{1,43} = 6.58$, $p < 0.02$; STR: $F_{1,33} = 4.93$, $p < 0.04$). For PCP-treated animals, but not controls (Figure 4), there was a significant negative correlation between serum glycine levels and potentiated amphetamine-induced DA release during the 150–210 min period ($r = -0.52$, $p < 0.03$), such that animals with serum glycine levels > 600 µM showed significantly lower levels of amphetamine-induced dopamine release than those with levels < 600 µM ($t = 2.91$, $p = 0.01$).

Glycine Transport Inhibitors

A final series of experiments evaluated the effects of the prototype glycine transport inhibitor NFPS. Owing to limited information concerning its brain penetration, NFPS was administered both intracerebroventricularly (i.c.v.) and systemically in separate studies. For i.c.v. studies, NFPS was administered via an osmotic minipump using a brain perfusion cannula inserted into the lateral ventricle. A dose

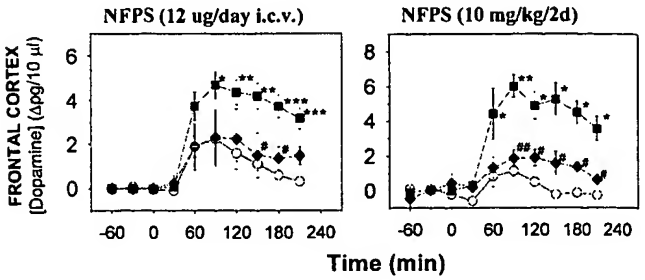

Figure 5 Effect of NFPS on amphetamine-induced DA release following i.c.v (left) or systemic (right) administration. *Left panel*: Amphetamine-induced DA release over basal (−90 to 0 min) in animals receiving NFPS for 2 weeks along with 15 mg/kg/day PCP (shaded circles) *vs* those receiving saline (open circles) or PCP alone (filled squares) $^{*}p < 0.05$ PCP *vs* saline; $^{**}p < 0.01$; $\#p < 0.05$ PCP + NFPS *vs* PCP alone. *Right panel*: amphetamine-induced DA release over the basal level was significantly lower in animals receiving systemic NFPS for 3 days with (shaded diamonds) or without 20 mg/kg/day PCP (open circles) *vs* those receiving PCP alone (filled squares). $^{*}p < 0.05$ PCP *vs* saline; $^{**}p < 0.01$, $^{***}p < 0.001$; $\#p < 0.05$ PCP + NFPS *vs* PCP alone, $\#\#p < 0.01$.

of 12 µg/day was used, representing the maximum dosage that could be achieved using this delivery system. For systemic studies, NFPS was administered at a dose of 10 mg/kg i.p. every other day (10 mg/kg/2 day). This dose is reported to induce a two-fold increase in brain glycine concentrations (Atkinson *et al*, 2001). A 3-day treatment regimen was used.

The amphetamine-induced DA release over basal was significantly lower in animals receiving NFPS for 2 weeks along with 15 mg/kg/day PCP *vs* those receiving PCP alone ($F_{9,162} = 2.2$, $p < 0.03$), but not significantly different than in animals receiving saline treatment alone ($F_{9,108} = 0.6$, $p > 0.8$). Animals treated with NFPS systemically ($F_{9,45} = 5.5$, $p < 0.001$) also showed significantly reduced amphetamine-induced dopamine release relative to animals that received PCP alone. The differences between animals treated with PCP and those treated with NFPS along with PCP were significant throughout the 90–210 min interval (Figure 5).

DISCUSSION

NMDA antagonists induce dopaminergic hyperactivity similar to that observed in schizophrenia in both human (Kegeles *et al*, 2000) and rodent (Balla *et al*, 2001b, 2003; Miller and Abercrombie, 1996) studies. In schizophrenia, treatment with NMDA co-agonists such as glycine, D-serine, and D-cycloserine produce significant amelioration of treatment-refractory symptoms, including improvement in both negative and positive symptoms (Javitt, 2002). Glycine effects are observed at serum levels of > 600 µM (Heresco-Levy *et al*, 1999; Leiderman *et al*, 1996). The present study demonstrates that glycine, at similar serum levels, reverses PCP-induced dysregulation of dopamine release in both the PFC and striatum, supporting the concepts first that it is effective in potentiating brain NMDA receptor-mediated neurotransmission and, second, that potentiation of NMDA receptor-mediated neurotransmission may be beneficial in schizophrenia.

The present results can be interpreted on both a neurochemical and clinical level. On the neurochemical level, these results are consistent with other studies showing the regulation of frontal and striatal DA systems by NMDA receptors. For example, Svensson (2000) has demonstrated that NMDA antagonists induce alteration in the firing pattern of neurons in the ventral tegmental area projecting to the PFC. Similarly, mice lacking the NR1 subunit show dysregulated dopaminergic systems (Miyamoto *et al*, 2001). We have previously observed that systemic PCP does not potentiate DA release induced by local amphetamine administration (Balla *et al*, 2001a), suggesting circuit level or other interactions. Hippocampal glutamatergic afferents may also regulate firing of midbrain DA neurons via both NMDA and non-NMDA afferents (Floresco *et al*, 2001). Thus, the critical sites of interaction in the present study may not be within the PFC or striatum, but may be within regions such as the ventral tegmental area and substantia nigra that project to these regions. Following single-dose administration, NMDA antagonists such as PCP or MK-801 stimulate DA release for 1–2 h (Javitt *et al*, 1999). In the present study, no sustained elevation in the basal DA levels was observed following long-term administration, suggesting that tolerance develops to this effect during chronic continuous administration.

On a clinical level, the present results may help explain the beneficial effects of NMDA agonists on symptoms of schizophrenia. Initial studies of NMDA agonists focused on the effect of these agents on persistent negative symptoms (Javitt, 2002). More recent studies, however, have documented the effects of these agents even on persistent positive symptoms in patients receiving typical or atypical antipsychotic treatment (Heresco-Levy *et al*, 2003; Tsai *et al*, 1998). Excess dopamine release in striatum has been shown to be associated with increased positive symptoms in amphetamine-challenged schizophrenic patients (Laruelle *et al*, 1996). To the extent that dopaminergic dysregulation in schizophrenia is due to underlying NMDA dysfunction, the ability of NMDA agonists to decrease amphetamine-stimulated DA release is consistent with the clinical therapeutic effect of these agents. In the present study, glycine was administered along with PCP throughout the study and prevented PCP effects. Future studies will be needed to determine the degree to which glycine supplementation added during the course of PCP treatment can reverse already established dopaminergic hyper-reactivity.

In schizophrenia, negative symptoms are thought to reflect functional hypodopaminergia, especially in the prefrontal cortex (Davis *et al*, 1991; Moore *et al*, 1999). However, as yet no studies have demonstrated reduced dopamine levels. In the present study, no change in baseline dopamine levels were observed in the prefrontal cortex. This finding is consistent with observations in both rats (Jentsch *et al*, 1998) and monkeys (Jentsch *et al*, 1997) that tissue dopamine levels remain unchanged following chronic treatment with NMDA antagonists, such as ketamine, PCP, or MK-801, although turnover rates (determined by metabolite:dopamine ratios) increase. The present findings argue against disturbances in absolute extracellular dopamine levels in schizophrenia, although not against disturbances in dopamine turnover. Since NMDA and D1 receptors show mutual facilitatory interactions (eg Chen and Yang, 2002; Scott *et al*, 2002; Wang and O'Donnell, 2001), disturbances in dopaminergic neurotransmission may occur independent of changes in extracellular dopamine levels.

The finding of exaggerated PFC response to amphetamine challenge in PCP-treated animals is also consistent with recent observations in schizophrenia. The majority of amphetamine challenge studies in schizophrenia have been conducted primarily with neuroleptic-treated, stabilized patients. In such patients, amphetamine typically produces small, but consistent improvement in cognitive functioning (eg Daniel *et al*, 1991; Goldberg *et al*, 1991). In contrast, a recent study investigated the effects of the indirect dopamine agonist methylphenidate in both acute and stabilized patients using the Word Production Test (Szeszko *et al*, 1999), a putative marker of prefrontal functioning (Yurgelun-Todd *et al*, 1996). Acute-phase patients showed significantly lower response rates than stabilized patients. Further, both groups showed deterioration of performance during methylphenidate challenge, along with increasing conceptual disorganization. By contrast, methylphenidate typically improves the prefrontal performance to novel tasks in normal volunteers (Elliott *et al*, 1997; Mehta *et al*, 2000), supporting the concept that patients show increased susceptibility to the disorganizing effects of prefrontal hyperdopaminergia following psychostimulant administration.

NMDA antagonists induce working memory dysfunction similar to those observed in schizophrenia (Adler *et al*, 1998; Krystal *et al*, 2000, 1994; Umbricht *et al*, 2000). Further NMDA agonists decrease conceptual disorganization (Javitt, 2002), and potentially improve working memory performance in schizophrenia (Tsai *et al*, 1998). The present findings are thus consistent with a model in which persistent NMDA dysfunction produces a tonic level of working memory dysfunction, which is prone to further disruption by hyperdopaminergia induced by either acute decompensation or psychopharmacological agents.

Aside from demonstrating the effectiveness of glycine and glycine transport inhibitors against PCP-induced augmentation of amphetamine-stimulated dopamine release, the present study provides the first demonstration that administration of clinically relevant glycine doses in animals leads to significant elevation of brain glycine levels. The administration of an 8% glycine diet to rodents, which led to a two-fold increase in serum glycine levels, also led to a two-fold increase in microdialysate glycine levels and a 1.4-fold increase in microdialysate serine levels. As glycine enters the brain by passive diffusion across the blood–brain barrier, serum and brain glycine levels would be expected to equilibrate over time. In acute studies in humans, an 8-fold increase in serum glycine levels produced a two-fold increase in CSF levels 1.5 h after administration (D'Souza *et al*, 2000). Although the glycine elevation observed in that study was significant, the results of the present study suggest that the degree of elevation of brain glycine levels *vs* serum during chronic treatment may be substantially higher than has been observed in acute studies. Glycine was well-tolerated in rats even during 3-month administration, as has been noted previously (Shoham *et al*, 1999). The present study suggests that administering 8–16% by weight glycine-enriched diet may be an effective method for producing

serum glycine levels similar to those observed during clinical studies in humans.

The mean serum PCP level in this study is similar to that observed in other studies using chronic PCP administration (eg Proksch *et al*, 2000). Notably, because of the slow infusion rate used, peak serum concentrations were substantially below those encountered during i.v. (Proksch *et al*, 2000) or i.p. (Bailey and Guba, 1980) administration of even relatively low doses of PCP (eg 1–3 mg/kg). NMDA antagonists, such as PCP, may induce neurodegeneration of structures involved in this study (ie PFC, striatum) following repeated, acute high-dose administration, for example, 20 mg/kg i.v. given once per day for 5 days. Neurotoxic effects, moreover, are significantly more pronounced in female, than male, rats (Johnson *et al*, 1998). Previous studies with male rats using doses similar to those in the present study (eg 10 mg/kg/day) have failed to observe significant effects of PCP on behavioral sensitization (Johnson *et al*, 1998) or PCP receptor binding (Burke *et al*, 1995).

In summary, the present treatment approaches for schizophrenia are based primarily upon dopaminergic models of the disorder. Although many patients respond well to antidopaminergic therapies, the majority of patients show persistent symptomatology despite treatment with either typical or atypical antipsychotic agents. The present study supports clinical research demonstrating significant improvement in negative, positive, and cognitive symptoms of schizophrenia with NMDA agonists including glycine, D-serine, and D-cycloserine (Goff *et al*, 1999; Heresco-Levy *et al*, 1999; Javitt *et al*, 2001, 1994; Shoham *et al*, 2001; Tsai *et al*, 1998). A prior study with the first available glycine transport inhibitor, GDA, demonstrated its ability to reverse PCP-induced hyperactivity in rodents (Javitt *et al*, 1999; Javitt and Frusciante, 1997). This study is the first to examine the *in vivo* effects of a more selective glycine transport inhibitor, NFPS. The finding that glycine transport inhibitors exert *in vivo* effects similar to glycine or other NMDA agonists supports the hypothesis that these agents may be useful in the treatment of persistent symptoms of schizophrenia.

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Prepulse Inhibition Deficits of the Startle Reflex in Neonatal Ventral Hippocampal–Lesioned Rats: Reversal by Glycine and a Glycine Transporter Inhibitor

Gwenaëlle Le Pen, James Kew, Daniela Alberati, Edilio Borroni, Marie-Paule Heitz, and Jean-Luc Moreau

Background: *Neonatal ventral hippocampal (NVH) lesions in rats induce behavioral abnormalities at adulthood thought to simulate some aspects of the positive, negative, and cognitive deficits classically observed in schizophrenic patients. Such lesions induce a postpubertal emergence of prepulse inhibition (PPI) deficits of the startle reflex reminiscent of the sensorimotor gating deficits observed in a majority of schizophrenic patients. To study the potential involvement of the glycinergic neurotransmission in such deficits, we investigated the capacity of glycine (an obligatory* N-*methyl-*D*-aspartate [NMDA] receptor co-agonist) and ORG 24598 (a selective glycine transporter 1 inhibitor) to reverse NVH lesion–induced PPI deficits in rats.*

Methods: *Ibotenic acid was injected bilaterally into the ventral hippocampus of 7-day-old pups. Prepulse inhibition of the startle reflex was measured at adulthood.*

Results: *Glycine (.8 and 1.6 g/kg IP) and ORG 24598 (10 mg/kg IP) fully and partially reversed lesion-induced PPI deficits, respectively.*

Conclusions: *These findings confirm that an impaired glutamatergic neurotransmission may be responsible for PPI deficits exhibited by NVH-lesioned rats and support the hypoglutamatergic hypothesis of schizophrenia. They also suggest that drugs acting either directly at the NMDA receptor glycine site or indirectly on the glycine transporter 1 could offer promising targets for the development of novel therapies for schizophrenia.* Biol Psychiatry 2003;54:1162–1170 © *2003 Society of Biological Psychiatry*

Key Words: Schizophrenia, neonatal lesion, hippocampus, prepulse inhibition, glycine

Introduction

Schizophrenia is characterized by episodic positive symptoms, such as delusions, hallucinations, paranoia, and psychosis, and by persistent negative symptoms, such as flattened affect, impaired attention, social withdrawal, and cognitive impairments (Ban et al 1984). Schizophrenia is presently viewed as a complex and heterogeneous disease that is attributable to neurodevelopmental perturbations involving several neurotransmitters and neuromodulators. The dopamine hypothesis has dominated schizophrenia research for decades but is now yielding to a more diversified view, in which the interaction of several neurotransmitters in complex circuitry is under scrutiny. Diminished activity of glutamatergic pathways has also been proposed to contribute to the etiopathology of schizophrenia. This hypothesis originated from the observation that, in clinical investigations, *N*-methyl-D-aspartate (NMDA) antagonists, such as phencyclidine and ketamine, induce behaviors reflecting the positive and negative symptoms as well as cognitive impairments observed in schizophrenia (Coyle 1996; Javitt and Zuckin 1991; Krystal et al 1999; Tamminga et al 1998). Moreover, these drugs have been shown to exacerbate these symptoms in schizophrenic patients (Jentsch and Roth 1999; Steinpreis 1996). Recently, postmortem studies performed in schizophrenic patients have revealed some alterations in NMDA receptor expression in certain brain areas (Gao et al 2000; Meador-Woodruff and Healy 2000). In addition, with relevance to the putative neurodevelopmental component of schizophrenia, NMDA receptors are critical in guiding axons to their targets in development (Rakic et al 1994) and in the synaptic pruning observed in adolescence, which can coincide with the onset of schizophrenia (Feinberg 1990).

A neurodevelopmental animal model based on long-term consequences of neonatal ventral hippocampal (NVH) lesions in the rat has been proposed by Lipska and co-workers as offering some degree of face and construct validity with schizophrenia (Lipska and Weinberger 1993,

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2000; Lipska et al 1993, 1994). The neuropathologic changes in hippocampal formation after such lesions are more extensive than those reported in schizophrenia (for review, see Weinberger 1999). Nevertheless, NVH lesions in rats have been shown to induce postpubertal emergence of behavioral abnormalities thought to simulate some aspects of positive, negative, and cognitive symptoms classically observed in schizophrenic patients. First, NVH-lesioned rats exhibited hyperresponsiveness to stress, novelty, and dopamine agonists (Black et al 1998; Brake et al 1999; Flores et al 1996; Lipska et al 1993, 1995a). Such lesions also induce deficits in social behavior (Becker et al 1999; Sams-Dodd et al 1997), in reward sensitivity (Le Pen et al 2002), in spatial and associative learning and working memory (Chambers et al 1996; Le Pen et al 2000), and in social memory (Becker and Grecksch 2000). Moreover, recent studies have shown a perturbation of glutamatergic (GLU) neurotransmission in this animal model. Thus, in the hippocampal formation as well as in the frontal cortex of NVH-lesioned rats, a hypofunction of the GLU system was shown by measuring ex vivo amino acid release from tissue slices (Schroeder et al 1999). At the behavioral level, it has been shown that NVH-lesioned rats are hypersensitive to the locomotor stimulatory effects of the GLU receptor antagonists MK-801 (Al-Amin et al 2000, 2001) and phencyclidine hydrochloride (PCP) (Hori et al 2000; Kato et al 2000). In addition, a recent study has shown that novelty- and amphetamine-induced locomotor activity in NVH-lesioned rats were reduced by administration of glycine (GLY), an obligatory NMDA receptor co-agonist at a strychnine-insensitive recognition site (glycine B) (Kato et al 2001). Thus, agents contributing to enhance GLU transmission might be predicted to counteract abnormal behaviors observed in NVH-lesioned rats.

Prepulse inhibition (PPI) of the startle reflex refers to the reduction in startle magnitude produced by a low-intensity, nonstartling prepulse that immediately precedes the startling stimulus. Prepulse inhibition models a form of preattentional sensorimotor gating and is reduced in several psychiatric disorders, including schizophrenia (for review, see Braff et al 2001). In rodents, the use of a PPI paradigm for screening antipsychotics is now well established. Drug-induced PPI deficits can be reversed by both typical and atypical antipsychotics (for review, see Geyer et al 2001), whereas in schizophrenics, atypical drugs seem to be more efficacious (Kumari and Sharma 2002; Leumann et al 2002; Oranje et al 2002). Interestingly, NVH lesions induce a reliable postpubertal emergence of PPI deficits (Le Pen and Moreau 2002; Le Pen et al 2000; Lipska et al 1995b) that can be reversed by atypical antipsychotics (Le Pen and Moreau 2002). In this study, we have investigated the capacity of GLY to reverse these PPI deficits. Glycine concentrations at synaptic NMDA receptors might be regulated by the GLYT1 glycine transporters that are co-localized with NMDA receptors in the brain (Javitt and Frusciante 1997; Malandro and Kilberg 1996; Smith et al 1992). Thus, we have also investigated the ability of ORG 24598, a potent and selective inhibitor of GLYT1 (Brown et al 2001) to reverse NVH lesion–induced PPI deficits.

Methods and Materials

Surgery

Sprague-Dawley rats (BRL, Füllinsdorf, Switzerland) were obtained at 3–4 days of age as whole litters together with their mothers. They were kept on a 12-hour light/12-hour dark cycle (on: 6:00 AM; off: 6:00 PM) and fed ad libitum. All experimental procedures and protocols were approved by the local animal protection authorities. On the seventh day of age and at a body weight of 15–20 g, male pups within each litter were randomized to sham (n = 24 and n = 65, respectively, for glycine and ORG experiments) or lesion (n = 30 and n = 74, respectively, for glycine and ORG experiments) status, anesthetized by isoflurane inhalation (4% for induction and 1.5%–3% for maintenance) through a mask, mounted on a stereotaxic instrument (David Kopf Instruments, Tujunga, California) with an adapter for small animals (Harvard Apparatus Inc., Holliston, Massachusetts) and additionally taped on a heating pad placed on the platform. The skin overlying the skull was incised, and .3 μL of either ibotenic acid (Sigma, St. Louis, Missouri; 10 μg/μL) or artificial cerebrospinal fluid was bilaterally infused over a 2-min period by a microinfusion pump (PHD Programmable, Harvard Biosciences) with an injection cannula (.3 mm diameter) aimed at the ventral hippocampal formation (antero-posterior [AP] −3.0 mm, mediolateral [ML] ± 3.5 mm, and ventro-dorsal [VD] −5.0 mm relative to bregma). After infusion, the skin overlying the skull was sutured and the animals allowed to recover on a heating pad before being returned to their mother (female pups had been removed from the litter). Eighteen days after surgery (i.e., postnatal day 25), rats were weaned and housed four per cage. Behavioral tests occurred between 9:00 AM and 4:00 PM and were initiated after puberty at postnatal day 70.

PPI of Startle Reflex

The apparatus consisted of eight startle chambers (SR-LAB, San Diego Instruments, San Diego, California), each containing a transparent Plexiglas tube (diameter 8.2 cm, length 20 cm) mounted on a Plexiglas frame within a ventilated enclosure. Acoustic noise bursts were presented via a speaker mounted 24 cm above the tube. Throughout the session, a background noise level of 68 dB was maintained. A piezoelectric accelerometer mounted below the frame detected and transduced motion within the tube. Startle amplitudes were defined as the average of 100 1-msec stabilimeter readings collected from stimulus onset. Rats were run in squads of eight. Each rat was put into the PPI chamber for a 5-min acclimatization period with 68-dB background noise. Following this period, 10 startle pulses (120 dB, 40-msec duration) were presented with an average intertrial

interval of 15 sec. Then, no stimulus (background noise, 68 dB), prepulses alone (72, 76, 80, or 84 dB, 20-msec duration), startle pulses alone, and prepulses followed 80 msec later by startle pulses were presented six times randomly distributed over the next 20 min. The percentage of PPI induced by each prepulse intensity was calculated as 100[(SP−SPP)/SP], with SP being the average startle amplitude after the startle pulses and SPP being the average startle response after the combination of a certain prepulse and the startle pulse.

Drugs

Glycine (Fluka Chemical, Milwaukee, Wisconsin) was dissolved in Tween 80 (0.3%) in sodium chloride (NaCl) 0.9% and injected in a volume of 10 mL/kg intraperitoneally. The selective glycine transporter 1 inhibitor ORG 24598 (synthesized at Roche Basel, Switzerland) was dissolved in 7.5% gelatin-modified XF 25/.62% NaCl solution and administered IP in a volume of 5 mL/kg. Drugs and their solvents were injected 30 min before testing. In each experiment, drugs were administered by pseudorandomized design over one or two treatment cycles, with a minimum period of 2 weeks between two cycles. Thus, for glycine experiment all rats were tested twice in PPI paradigm, whereas for the ORG 24598 experiment some of the animals were tested once and the others twice.

Rating of Lesion Size

At the completion of behavioral evaluation, rats were killed by decapitation. Brains were rapidly removed, and after fixation in formalin solution (10% in NaCl), 40-μm sections were sliced with a freezing cryostat. The sections through the lesioned area were mounted and stained with cresyl violet. The extent of the lesion on each side of the brain was rated (according to Sams-Dodd et al 1997) as follows: 0 = no discernible cell loss in the hippocampal formation, 1 = small, 2 = medium, 3 = large area of cell loss within the ventral hippocampal formation. Scores for both sides were added, to yield a total score ranging from 0 to 6.

Analysis of Data

Prepulse inhibition data were analyzed with a three-factor analysis of variance (ANOVA) (lesion status × drug dose × prepulse intensity) with repeated measurements on factor prepulse intensity, followed when appropriate by separate two-factor ANOVA or by the Fisher protected least significant difference (PLSD) post hoc test. Startle amplitude data were analyzed with a two-factor ANOVA (lesion status × drug dose) followed when appropriate by post hoc comparisons (Fisher PLSD test).

Results

Histology

Subjects with unilateral or extrahippocampal damage were discarded from the study (GLY experiment: 3 and 5 rats, respectively; ORG 24598 experiment: 6 and 7 rats, respectively). Neonatally lesioned rats evaluated in PPI experiments with GLY and ORG 24598 exhibited a mean lesion score of 4.01 ± .50 and 3.61 ± .31, respectively, indicating that the cell loss was restricted to the ventral part of the hippocampus (Figure 1). Some animals exhibited cavitation around the site of injection. In control rats that had been injected with artificial cerebrospinal fluid, the hippocampus was morphologically intact (lesion score 0). It has to be noted that rats excluded after histology exhibited PPI deficits similar to those observed for rats included in the analysis.

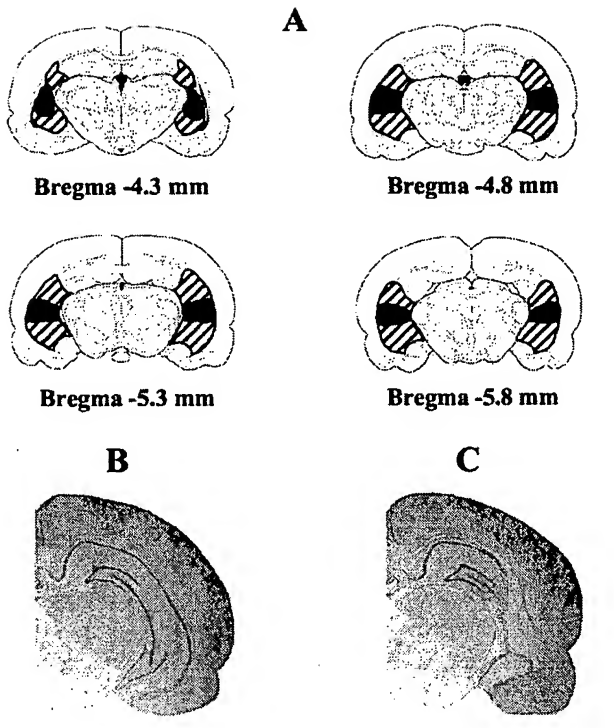

Figure 1. Lesion boundaries in the ventral hippocampus of adult rats infused bilaterally with ibotenic acid at postnatal day 7. **(A)** Schematic drawing of the ventral hippocampus with boundaries of the largest (stripes) and smallest (black) lesions. **(B,C)** Representative photomicrographs of Cresyl violet-stained coronal section through the brains of adult rats that had received neonatal sham **(B)** or ibotenic acid **(C)** lesions of the ventral hippocampus.

Prepulse Inhibition of the Startle Reflex

GLYCINE. Neonatal ventral hippocampal lesions induced significant PPI deficits, and these effects were reversed by GLY (Figure 2). A three-way ANOVA of PPI revealed a significant effect of the lesion [$F(1,82) = 10.2$, $p < .01$], a significant effect of GLY [$F(2,82) = 6.3$, $p < .01$], and a significant effect of prepulse intensity [$F(3,402) = 106.7$, $p < .001$]. The overall analysis also revealed a significant interaction lesion × GLY [$F(3,82)$

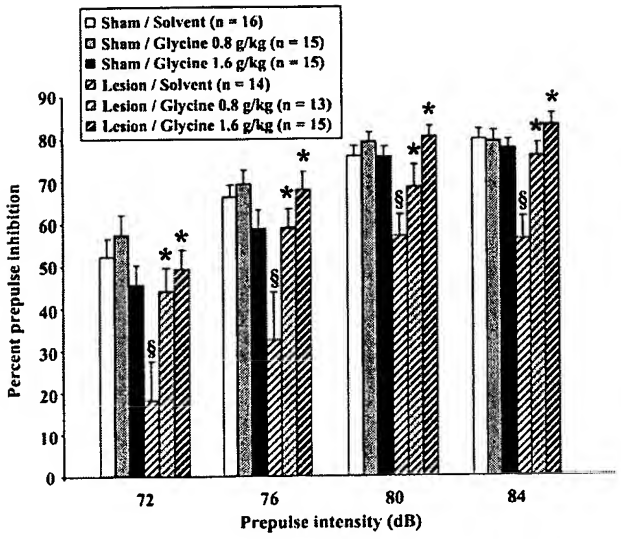

Figure 2. Effects of glycine on neonatal ventral hippocampal lesion–induced deficits in prepulse inhibition of startle reflex. A study using vehicle, .8, or 1.6 g/kg of glycine injected IP 30 min before testing was performed. § $p < .01$ compared with sham group. * $p < .001$ compared with lesion/vehicle group.

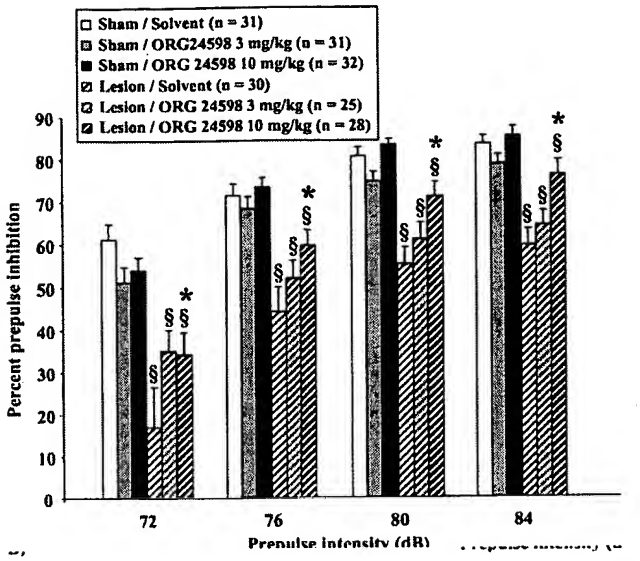

Figure 3. Effects of ORG 24598 on neonatal ventral hippocampal lesion–induced deficits in prepulse inhibition of startle reflex. A study using vehicle, 3, or 10 mg/kg of ORG 24598 injected IP 30 min before testing was performed. § $p < .001$ compared with sham group. * $p < .001$ compared with lesion/vehicle group.

$= 9.1, p < .001$], indicating that GLY had different effects in sham and lesioned animals. Finally, no other two- or three-way interactions were observed. Global post hoc comparisons revealed that GLY did not modify PPI in sham animals. Conversely, in NVH-lesioned rats, PPI was significantly greater in rats administered GLY (.8 and 1.6 g/kg), in which a full reversal of PPI deficits was observed, as compared with rats treated with solvent (both $p < .001$).

ORG 24598. Effects of ORG 24598 (0–30 mg/kg) on locomotor activity were noticed at 10 and 30 mg/kg (data not shown). At 10 mg/kg, a decrease of locomotion was observed during the first 30 min after injection. At 30 mg/kg, toxic effects (abnormal posture, difficulty breathing) were observed as much as 12 hours after the end of the test, when rats were found moribund in their home cages. Thus, in our experiments, a maximal dose of 10 mg/kg of ORG 24958 was used to allow repeated PPI testing in the same rats, by use of a semi-Latin square design.

Here also, neonatal ventral hippocampal lesions induced significant PPI deficits, and these effects were reversed by ORG 24598 (Figure 3). A three-way ANOVA of PPI revealed a significant effect of the lesion [$F(1,171) = 54.1$, $p < .0001$], a significant effect of ORG 24598 [$F(2,171) = 3.5$, $p < .05$], and a significant effect of prepulse intensity [$F(3,513) = 238.5$, $p < .0001$]. The overall analysis also revealed a significant interaction lesion × ORG 24598 [$F(2,171) = 3.9, p < .05$], indicating that ORG 24598 had different effects in sham and lesioned animals. In addition, a significant interaction of lesion × prepulse intensity [$F(3,513) = 6.8$, $p < .0001$] was observed, and no other two- or three-way interactions. Global post hoc comparisons revealed that ORG 24598 did not modify PPI in sham animals. Conversely, in NVH-lesioned rats, PPI was significantly greater in rats given ORG 24598 (10 mg/kg) as compared with vehicle-treated animals ($p < .001$) but never reach sham levels, indicating a partial reversal of observed PPI deficits at this dose. In addition, at 3 mg/kg, ORG 24598 tended to increase PPI ($p = .066$).

Startle Amplitude

Drug effects on startle amplitude (pulse alone) are seen in Figure 4. In the glycine experiment (Figure 4, left panel), we replicated the previous findings that lesion has no effect on startle amplitude [$F(1,82) = 3.5$, ns] (Le Pen and Moreau 2002; Le Pen et al 2000; Lipska et al 1995b). In contrast to the glycine experiment, a significant overall lesion effect was observed in the ORG 24598 experiment [$F(1,171) = 10.4$, $p < .01$] (Figure 4, right panel).

Moreover, in both the GLY and ORG 24598 experiments (Figure 4, left and right panels, respectively), an overall effect of drug was observed on startle amplitude [$F(2,82) = 3.8, p < .05$ and $F(2,171) = 11.1, p < .001$, respectively], but no significant lesion × drug interaction could be detected. Post hoc comparisons revealed that ORG 24598 (10 mg/kg) induced a significant decrease and a trend to decrease startle amplitude in sham and lesioned

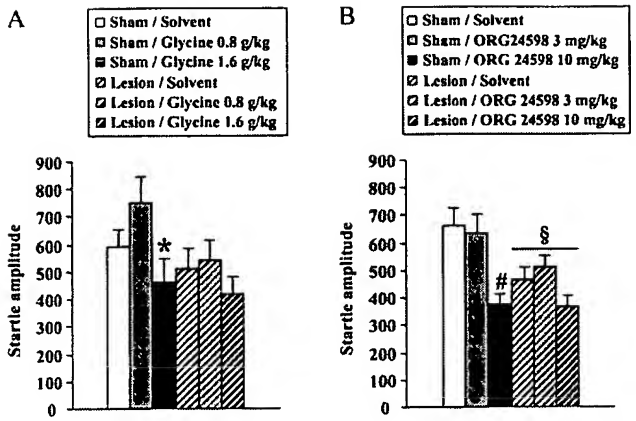

Figure 4. Effects of glycine **(A)** and ORG 24598 **(B)** on startle amplitude in sham-operated and neonatal ventral hippocampal–lesioned rats. § $p < .001$ compared with sham group. # $p < .001$ at least compared with respective sham/vehicle group. * $p < .01$ compared with sham/glycine .8 g/kg group.

animals, respectively. In the GLY experiment, post hoc comparisons revealed that the overall GLY effect was due to a difference of startle at .8 and 1.6 g/kg in sham animals.

Discussion

In good agreement with previous reports (Le Pen and Moreau 2002; Le Pen et al 2000; Lipska et al 1995b), deficits in PPI of the startle reflex have been observed in rats after NVH lesions. These deficits are reminiscent of those commonly observed in schizophrenic patients (Braff et al 1978; Kumari et al 2000; Parwani et al 2000).

Early maternal deprivation has been shown to induce PPI deficits in rats (Ellenbroek et al 1998). In addition, offspring of mothers that show poor levels of pup licking and grooming also exhibit poor spatial learning and locomotor performances (Gomez-Serrano et al 2001; Liu et al 2000). According to these results, PPI deficits seen in NVH-lesioned rats could result from reduced maternal care. A recent study of the behavioral outcome of NVH lesions in two strains of rats exhibiting differences in the frequency of maternal behavior has revealed no difference of maternal care toward lesioned or control pups (Wood et al 2001). Nevertheless, NVH-lesioned rats were significantly more affected by increases in arched-back nursing compared with control animals (Wood et al 2001). Thus, increased maternal interactions could actually lead to some of the deficits seen in NVH-lesioned rats.

High doses of glycine (GLY), an obligatory NMDA receptor co-agonist, induced a full reversal of NVH lesion–induced PPI deficits, whereas the glycine transporter 1 (GLYT1) inhibitor ORG 24598 only produced a partial reversal. The effects of GLY and GLYT1 inhibitor on sensory motor gating deficits have not been investigated previously in animals or in man. In rodents, systemic injections of NMDA receptor glycine site agonists, such as GLY and D-serine, have been shown to inhibit hyperactivity and stereotypy elicited by PCP or MK-801 (Contreras 1990; Javitt et al 1997; Javitt and Frusciante 1997; Nilsson et al 1997; Riekkinen et al 1998; Tanii et al 1994). Moreover, glycyldodecylamide, a systematically active GLYT1 inhibitor, was found to potently reverse PCP-induced hyperactivity in rodents (Javitt and Frusciante 1997). Recently, GLY has been shown to reduce novelty- and methamphetamine-induced hyperlocomotion in NVH-lesioned rats (Kato et al 2001). Glycine site agonists have also been shown to improve cognitive deficits observed after scopolamine injections (Fishkin et al 1993; Ohno and Watanabe 1996; Sirvio et al 1992), hippocampal lesions (Schuster and Schmidt 1992), or entorhinal cortex lesions (Myhrer 1992; Myhrer and Paulsen 1992).

In humans, glycine site agonists have been shown to reduce negative symptoms and improve cognitive function in treatment-resistant schizophrenic patients receiving typical or atypical antipsychotics (Evins et al 2002; Heresco-Levy et al 1996, 1999; Javitt et al 1994). Thus, these preclinical and clinical data argue that glycine enhancement may provide a promising therapy for schizophrenia.

It is worth noting that the inability of ORG 2498 to fully reverse PPI deficits observed in NVH-lesioned rats is unlikely to be simply a consequence of its reduction of startle. Indeed, as previously stated, there is no consistent relationship between the startle amplitude value and the effectiveness of a given drug to restore PPI deficits (Le Pen and Moreau 2002; Schwarzkopf et al 1992; Swerdlow et al 1996; Varty and Higgins 1995).

Glycine- and GLYT1 inhibitor–induced improvement of PPI deficits in NVH-lesioned rats suggests a dysfunction of NMDA receptor function in these animals. Indeed, numerous data directly suggest a down-regulation of the glutamatergic activity in NVH-lesioned rats (see Introduction). In addition, we have recently shown that atypical antipsychotics, but not the typical antipsychotic haloperidol, are able to reverse PPI deficits observed in NVH-lesioned rats (Le Pen and Moreau 2002). This last result may also suggest indirectly that NVH lesion–induced PPI disruptions may be more dependent on GLU than on dopamine mechanisms. Indeed, clozapine, olanzapine, or risperidone, in contrast to haloperidol, have been shown to attenuate PCP-induced reduction of PPI in rats (Bakshi and Geyer 1995; Bakshi et al 1994; Keith et al 1991; Swerdlow et al 1996; Varty and Higgins 1995; Yamada et al 1999). Together, these results suggest that NVH lesion–induced PPI deficits could result from a dysfunctioning of the glutamate system, which might be alleviated by atypical antipsychotics.

Interestingly, no PPI deficits have been observed after adult VH lesions (Swerdlow et al 1995, 2000), suggesting that NVH lesions may affect anatomic substrates for PPI other than the hippocampus, such as the medial prefrontal cortex (mPFC) or nucleus accumbens (NA). These two structures are involved in the circuitry of prepulse inhibition (Swerdlow et al 2001), and both receive direct GLU projections from the ventral hippocampus (Brog et al 1993; Fink 1993; Groenewegen et al 1987; Jay et al 1996). Moreover, in NVH-lesioned rats, GLU dysfunction within the mPFC or NA might parallel data obtained in the clinic. Hypoactivity of the mPFC, in which GLU acts as a major neurotransmitter, is considered as a relevant parameter for cognitive dysfunction in schizophrenic patients (Carlsson and Carlsson 1990; Hirsch et al 1997; Tsai and Coyle 2002; Weinberger 1997). In addition, levels of GLU and expression of its receptors have been shown to be altered in the cortex of schizophrenic subjects (Akbarian et al 1996; Meodor-Woodruff and Healy 2000). Proton magnetic resonance spectroscopy experiments have revealed a reduction of GLU activity in the mPFC of nonmedicated schizophrenic patients (Bartha et al 1997). In addition, an increase of glycine binding sites was observed in the cerebral cortex of chronic schizophrenic patients, possibly reflecting a compensatory mechanism for impaired GLU transmission (Ishimaru et al 1994). Finally, the density of GLU uptake sites is decreased in the NA of schizophrenic patients (Aparicio-Legarza et al 1997). Thus, NVH lesions could impair these connections as well as GLU functions in the PFC and NA and lead to PPI deficits.

This hypothesis is further substantiated by the fact that high doses of NMDA antagonists infused into the NA have been shown to induce PPI deficits (Reijmers et al 1995). More interestingly, NMDA receptors in NA could play an important role in sensorimotor information processing without affecting mesolimbic dopamine transmission. Indeed, intra-accumbens injections of 7-chlorokynurenate, an NMDA receptor glycine site antagonist, has been shown to reduce PPI independently of an increase of dopamine release in NA or striatum. This deficit is reversed by systemic injection of the glycine site agonist D-cycloserine (Kretschmer and Koch 1997).

In addition, NVH lesions induced a reduction in the number of neurons in prefrontal cortex and perirhinal and enthorhinal cortical regions (Bernstein et al 1999), two regions in which information originating from most sensory modalities converges first before reaching the hippocampus (Deacon et al 1983). Entorhinal lesions have been shown to disrupt PPI (Goto et al 2002); however, these deficits were reversed by haloperidol (Goto et al 2002), suggesting that NVH lesion–induced PPI deficits are unlikely due to a reduction in the number of neurons in the entorhinal cortex.

Finally, the basolateral amygdala (BLA), a structure that receives projections from the ventral hippocampus (Pitkanen et al 2000; Van Groen and Wyss 1990), could also be implicated in PPI deficits exhibited by NVH-lesioned rats. It has been shown that neonatal BLA lesions induced abnormal behaviors similar to those produced by NVH lesions, such as deficits in PPI (Bouwmeester et al 2002; Daenen et al 2001; Wolterink et al 2001). Moreover, lesions of BLA or infusion of NMDA antagonists (MK-801 or AP-5) into the BLA have been reported to disrupt PPI (Bakshi and Geyer 1998; Wan and Swerdlow 1997) in a haloperidol-reversible manner (Fendt et al 2000). Thus, PPI deficits induced by NVH lesions are unlikely to be only a consequence of a glutamatergic dysfunctioning in the BLA.

In conclusion, our results demonstrate that GLY and a GLYT1 inhibitor (ORG 24598) were able to reverse NVH lesion–induced PPI deficits, in good agreement with the hypoglutamatergic hypothesis of schizophrenia. These results suggest that an impaired glutamatergic innervation of cortical and subcortical regions could be responsible for PPI deficits exhibited by NVH-lesioned rats. Finally, these findings also suggest that agents acting directly at the NMDA receptor glycine site or indirectly through an action at GLYT1 could offer promising targets for the development of novel therapies for schizophrenia.

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Glycine Transporter I Inhibitor, N-Methylglycine (Sarcosine), Added to Antipsychotics for the Treatment of Schizophrenia

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Background: *Hypofunction of N-methyl-D-aspartate glutamate receptor had been implicated in the pathophysiology of schizophrenia. Treatment with D-serine or glycine, endogenous full agonists of the glycine site of N-methyl-D-aspartate receptor, or D-cycloserine, a partial agonist, improve the symptoms of schizophrenia. N-methylglycine (sarcosine) is an endogenous antagonist of glycine transporter-1, which potentiates glycine's action on N-methyl-D-aspartate glycine site and can have beneficial effects on schizophrenia.*

Methods: *Thirty-eight schizophrenic patients were enrolled in a 6-week double-blind, placebo-controlled trial of sarcosine (2 g/d), which was added to their stable antipsychotic regimens. Twenty of them received risperidone. Measures of clinical efficacy and side effects were determined every other week.*

Results: *Patient who received sarcosine treatment revealed significant improvements in their positive, negative, cognitive, and general psychiatric symptoms. Similar therapeutic effects were observed when only risperidone-treated patients were analyzed. Sarcosine was well-tolerated, and no significant side effect was noted.*

Conclusions: *Sarcosine treatment can benefit schizophrenic patients treated by antipsychotics including risperidone. The significant improvement with the sarcosine further supports the hypothesis of N-methyl-D-aspartate receptor hypofunction in schizophrenia. Glycine transporter-1 is a novel target for the pharmacotherapy to enhance N-methyl-D-aspartate function.*

Key Words: Glutamate, GlyT-1, N-methyl-D-aspartate, sarcosine, schizophrenia, treatment

There is a great need to develop new antipsychotic agents, providing additional benefits for the substantial portion of schizophrenic patients who are only partially responsive or resistant to the available antipsychotics. In addition to dopaminergic neurotransmission, glutamatergic neurotransmission has been implicated in the pathophysiology of schizophrenia (Tsai and Coyle 2001; Olney and Farber 1995). N-methyl-D-aspartate (NMDA) receptor, a subtype of ionotropic glutamate receptor, plays an important role in neurocognition and neurotoxicity. Glutamate and glycine serve as coagonists at the NMDA receptor with activation of both the glutamate and glycine sites required for channel opening (Thomson et al 1989). Glycine is present at micromolar levels in the cerebrospinal fluid (CSF), a concentration that would appear to saturate the site. But the glycine transporter-1 (GlyT-1) plays a pivotal role in maintaining the concentration of glycine within synapses at a subsaturating level. Supporting the critical role GlyT-1 plays in NMDA neurotransmission, GlyT-1 inhibitor, a sarcosine analogue, N[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)propyl] sarcosine can enhance the NMDA neurotransmission (Bergeron et al 1998a; Chen et al 2003). In behavioral studies, the potency of a series of GlyT-1 antagonists for inhibiting phencyclidine (PCP)-induced hyperactivity in vivo correlated significantly with their potency in antagonizing GlyT-1 in vitro (Javitt et al 1999). Also, the anatomical distribution of GlyT-1 is parallel to the NMDA receptor (Smith et al 1992).

The most compelling link between the NMDA system and schizophrenia concerns the mechanism of action of phencyclidine; for reviews, see Tsai Coyle 2001; Halberstadt 1995; Javitt and Zukin 1991. The psychotomimetic PCP and its analogues ketamine and MK-801 bind to a site within the channel and act as noncompetitive antagonists. Use of PCP, ketamine, or MK-801 can produce a psychotic condition with compelling similarities to schizophrenic psychosis (Grotta 1994; Herrling 1994; Javitt and Zukin 1991; Kristensen et al 1992; Krystal et al 1994).

In fact, NMDA antagonist-induced psychosis more closely resembles schizophrenia than the amphetamine/dopamine agonist model (Krystal et al 1994). The psychosis induced by NMDA antagonists causes not only positive symptoms similar to the action of dopaminergic agonists but also negative symptoms and cognitive deficits associated with schizophrenia. The ability of PCP or ketamine to induce a syndrome closely resembling schizophrenia by inhibiting NMDA receptor-mediated neurotransmission suggests that dysfunction or dysregulation of NMDA receptor-mediated neurotransmission might be a critical deficit in schizophrenia (Tsai and Coyle 2001; Olney and Farber 1995). Accordingly, potentiation of NMDA receptor-mediated neurotransmission has been investigated as a treatment of schizophrenia.

Excessive stimulation of NMDA receptor can cause neuronal toxicity by a process called "excitotoxicity." But enhancing NMDA neurotransmission via the glycine site is safer than the NMDA site (Farber et al 1999). Several studies have demonstrated the clinical benefits of treatment for schizophrenia targeting the glycine site of the NMDA receptor (NMDA-glycine site) and no significant side effect was noted. In fact, these agents improve the cognitive symptoms. Nevertheless, subtle, but clinically insignificant, neuronal toxicity cannot be ruled out. This included D-serine (Tsai et al 1998b), glycine (Heresco-Levy et al 1999),

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Table 1. Characteristics of Schizophrenic Patients Assigned to Placebo and Sarcosine Treatment

	Sarcosine (n = 17)	Placebo (n = 21)	(t, p)[a]
Gender (female/male)	7/10	8/13	*ns*
Age (years)	29.8 (7.2)	33.4 (8.3)	*ns*
Education (years)	11.8 (2.5)	11.3 (3.6)	*ns*
Age of Onset (years)	19.4 (4.5)	24.5 (6.8)	(2.68, .01)
Duration of Illness (years)	10.5 (5.6)	8.9 (5.6)	*ns*
Subtype			
Paranoid	7	12	
Disorganized	2	1	
Undifferentiated	4	5	
Residual	4	3	
Chlorpromazine Equivalence (mg)[b]	409 (320)	433 (243)	*ns*
Risperidone (mg)[c]	3.7 (1.6)	4.9 (2.0)	*ns*

Standard deviations in parentheses.

[a]As assessed by two-sample t test or χ^2 test where appropriate, all df = (1, 36).

[b]Risperidone dosages were calculated as 1 mg = 75 mg chlorpromazine.

[c]Placebo group n = 11, sarcosine group n = 9.

and D-cycloserine (Goff et al 1999; Heresco-Levy et al 2002). Another approach to enhance the NMDA neurotransmission is through increasing the availability of synaptic glycine by the attenuation of the glycine reuptake through GlyT-1. N-methyl glycine (sarcosine) is a potent endogenous antagonist at the GlyT-1 site (McBain et al 1989). Sarcosine is also a methyl donor, and there is no known neurotransmitter system affected by sarcosine. For the present study, we hypothesized that sarcosine is a therapeutic agent for schizophrenia due to its activity as an antagonist on the GlyT-1.

Methods and Materials

Subjects

Patients were recruited from the day program and inpatient units of Taipei City Psychiatry Center and Kaoshiung Medical University, which are major medical centers in Taiwan. The research protocol was approved by the above Institutional Review Boards (IRBs). Sarcosine is a natural amino acid. It is regulated as food supplement and investigational new drug application (IND) is not required in Taiwan. After a description of the study to the patients, written informed consent was obtained. Thirty-eight schizophrenic patients enrolled and 36 patients completed the double-blind, placebo-controlled study. One patient each in the placebo and sarcosine groups dropped out after week 4 assessment due to poor compliance. Demographic information of the patients is shown in Table 1.

Patients were evaluated by the research psychiatrists after a thorough medical and neurologic workup. The Structured Clinical Interview for DSM-IV (American Psychiatric Association 1994b) was conducted for the diagnosis. Patients with an axis I diagnosis other than schizophrenia, significant depressive symptoms (Hamilton Depression Scale > 25), or a serious medical or neurologic illness were not included. All the enrolled patients had a normal physical examination, neurologic examination, and laboratory screening tests.

All enrolled patients fulfilled the DSM-IV diagnosis of schizophrenia (American Psychiatric Association 1994a). Their antipsychotic doses had remained stable for at least 3 months before enrollment in the study, and they remained on the same antipsychotic regimen for the period of the sarcosine trial. The antipsychotics received by the patients included risperidone (n = 20), sulpiride (n = 6), haloperidol (n = 5), sulpiride and chlorpromazine (n = 1), pipotiazine and chlorpromazine (n = 1), chlorpromazine (n = 1), fluphenazine decanoate (n = 1), trifluoperazine (n = 1), etumine (n = 1), and medication free (n = 1).

A separate open-label, pilot dose-finding trial was performed first by escalating the sarcosine dose from 10 to 30 mg/kg/d (n = 3). All patients were randomly assigned under double-blind conditions to receive a 6-week trial of placebo or 2 g of sarcosine daily (body weight 65.1 ± 12.8 kg, ~30 mg/kg/d). Sarcosine was provided by Natural Pharmacia International, Inc. (Belmont, Massachusetts). Purity of more than 99% was confirmed by high-performance liquid chromatography (HPLC). Placebo and sarcosine were packed with the same additives and capsules.

Assessments

Baseline scales were performed as follows: Positive and Negative Syndrome Scale (PANSS) (Kay et al 1987; Lindenmayer et al 1994), Scales for the Assessment of Negative Symptoms (SANS) (Andreasen 1983), Brief Psychiatric Rating Scale (BPRS) (items extracted from the PANSS and score 0–6) (Kay et al 1987), and Hamilton Depression Rating Scale (Hamilton) (Hamilton 1960). Clinical ratings were performed by research psychiatrists who were trained and experienced in the rating scales. The interrater reliability was ≥ .9. Individual patients were assessed by the same research psychiatrist throughout the trial. All assessments were blind to treatment assignment and completed at baseline and at the end of every 2-week period.

Biweekly side effect assessments included Simpson-Angus Rating Scale for extrapyramidal side effects (Simpson and Angus 1970b), Abnormal Involuntary Movement Scale (AIMS) for dyskinesia (Guy 1976), and Barnes Akathesia Scale (Barnes 1989). Systemic side effects of sarcosine treatments were reviewed by applying the Udvalg for Kliniske Undersogelser (UKU) Side Effects Rating Scale (Lingjaerde et al 1987).

Data Analysis

Demographic characteristics between groups were compared by Student two-sample t tests for continuous variables and by χ^2 tests for categorical variables. For efficacy assessment and possible side effects, linear mixed-effects models (Lange and Ryan 1989) were fit to all normally distributed outcomes, with main effects for time and treatment (sarcosine or placebo), time (0, 2, 4, and 6 weeks), and the treatment-by-time interaction. Significance of treatment effects over time was assessed by the significance of the treatment-by-time interaction while controlling for the main effects. All hypothesis tests were two-sided and conducted at the .05 α level. Results of the fitted models were also re-expressed in equivalent repeated measures analysis of variance (ANOVA) tables.

Results

Characteristics of the schizophrenic illness were similar in the two groups, except the onset of the illness (Table 1). Both groups had similar ratios of paranoid versus nonparanoid subtypes of schizophrenia. Though the sarcosine group has earlier onset of the illness than the placebo group, the sarcosine group improved in the outcome measures, but the placebo group did not improve.

Table 2. Clinical Measures for the 6 Week Placebo-Controlled Sarcosine Trial

Scale	Treatment	Baseline	Week 2	Week 4	Week 6	ANOVA (F, p)			Mixed Regression Coefficient t	
						Treatment	Time	Treatment X Time	SE	p
SANS	Sarcosine	53.1 (14.7)	49.6 (16.7)	46.8 (18.0)	45.6 (18.6)	.05	38.2	23.7	−1.08	−4.87
	Placebo	50.4 (13.7)	50.2 (15.3)	49.9 (14.1)	49.2 (14.1)	.83	.0003	< .0001	.22	< .0001
Positive	Sarcosine	18.2 (5.5)	16.7 (6.1)	15.8 (5.9)	15.1 (5.8)	.32	35.6	17.9	−.42	−4.23
	Placebo	17.9 (5.5)	17.3 (5.3)	17.6 (5.2)	17.1 (5.0)	.57	< .0001	.0006	.10	< .0001
Cognitive	Sarcosine	14.2 (5.0)	13.6 (4.8)	13.1 (4.6)	12.4 (4.4)	.01	33.3	32.2	−.29	−5.68
	Placebo	13.3 (3.3)	13.1 (3.4)	13.3 (3.6)	13.3 (3.6)	.93	< .0001	< .0001	.05	< .0001
General	Sarcosine	37.8 (9.6)	35.3 (10.5)	33.4 (10.5)	32.7 (9.9)	5.31	25.0	15.0	.74	−3.87
	Placebo	41.4 (4.8)	40.9 (6.1)	41.2 (7.9)	40.4 (8.0)	.03	< .0001	.0002	.19	.0002
Total	Sarcosine	82.6 (17.7)	77.3 (20.2)	73.6 (21.0)	71.4 (20.5)	2.65	47.5	26.4	−1.57	−5.14
	Placebo	85.2 (7.2)	83.8 (9.2)	84.4 (10.7)	82.8 (10.7)	.11	< .0001	< .0001	.30	< .0001
BPRS	Sarcosine	36.9 (10.2)	34.5 (10.7)	32.3 (10.8)	31.2 (11.2)	.42	35.9	16.5	.78	−4.06
	Placebo	32.3 (10.1)	31.6 (9.3)	31.9 (9.9)	30.9 (9.9)	.52	< .0001	.0001	.19	.0001

Standard deviations in parentheses. Repeated measures analysis of variance (ANOVA) with factors of treatment (sarcosine or placebo) and time (week 0, 2, 4, 6) were applied to determine the significance of observed treatment (df = 1,36), time (df = 1,110), and treatment by time effects (df = 1,110). Outcome measures are also analyzed by linear mixed-effects regression model (coefficient = estimated coefficient, SE = standard error). Positive, cognitive, general, and total are from the Positive Symptom Subscale of the Positive and Negative Syndrome Scale.

BPRS, Brief Psychiatric Rating Scale; SANS, Scales for the Assessment of Negative Symptoms.

Clinical Outcomes

The results of ANOVA and mixed regression are presented in Table 2. The positive symptoms showed significant improvement with sarcosine treatment (Table 2) [PANSS-positive subscale, ANOVA treatment × time, $F(1,110) = 17.9$, $p < .0001$; mixed regression, $t = -4.23$, $p < .0001$]. At the end of the 6-week trial, sarcosine treatment resulted in a 17% reduction of the positive symptoms and a 14% reduction of the negative symptoms [SANS, $F(1,110) = 23.7$, $p < .0001$; mixed regression, $t = -4.87$, $p < .0001$]. Scores of PANSS-cognitive subscale also indicated a significant 13% improvement in the sarcosine group (Table 2) [$F(1,110) = 32.2$, $p < .0001$; mixed regression, $t = -5.68$, $p < .0001$].

The scores of PANSS-general subscale (14% improvement, $F = 15.0$, $p = .0002$; mixed regression, $t = -3.87$, $p = .0002$), BPRS (16% improvement, $F = 16.5$, $p = .0001$; mixed regression, $t = -4.06$, $p = .0001$) and PANSS-total (14% improvement, $F = 26.4$, $p < .0001$; mixed regression, $t = -5.14$, $p < .0001$) were all improved in the sarcosine group, and no significant reduction in symptoms of any type were present in the placebo group (Table 2). The scores of the Hamilton Depression Rating Scale were low in both groups and were similar following 6 weeks of sarcosine or placebo treatments (sarcosine group from baseline of 4.6 ± 5.5 to week 6 of 3.1 ± 4.5; placebo group from baseline of 6.6 ± 4.6 to week 6 of 5.9 ± 6.2). Overall, nearly half (8 out of 17) sarcosine-treated patients had more than 20% improvement of their BPRS scores.

When analyzing the risperidone-treated patients alone (11 placebo and 9 sarcosine-treated), similar results were found; significant improvement of positive symptoms with sarcosine treatment in the risperidone-treated patients [17%, PANSS-positive subscale, ANOVA treatment × time, $F(1,57) = 12.7$, $p = .0007$; mixed regression, $t = -3.56$, $p = .0007$]. At the end of the 6-week trial, sarcosine treatment resulted in a 14% reduction of the negative symptoms (SANS, $F = 10.2$, $p = .002$; mixed regression, $t = -3.19$, $p = .002$). Scores of PANSS-cognitive subscale also indicated a significant 16% improvement in the sarcosine group ($F = 15.9$, $p = .0002$; mixed regression, $t = -3.99$, $p = .002$). The scores of PANSS-general subscale (17% improvement, $F = 8.3$, $p = .006$; mixed regression, $t = -2.89$, $p = .006$), BPRS (18% improvement, $F = 13.8$, $p = .0005$; mixed regression, $t = -3.72$, $p = .0005$), PANSS-total scale (15% improvement, $F = 13.6$, $p = .0005$; mixed regression, $t = -3.69$, $p = .0005$) were improved in the sarcosine group of risperidone-treated patients, and no significant reduction in symptoms of any type were present in the placebo group.

Side Effects

Both the sarcosine and placebo groups had mild extrapyramidal symptoms at the beginning of the study. The baseline scores of Simpson-Angus (sarcosine group 1.1 ± 1.6, placebo group 2.1 ± 2.2), AIMS (sarcosine group .2 ± 1.0, placebo group .3 ± .7), and Barnes Akathesia Scale (sarcosine group .1 ± .5, placebo group .0 ± .0) were similar in both groups. Both the placebo and sarcosine treatment groups did not change their profiles of side effects and were not different from each other through the 6 weeks (Simpson-Angus, sarcosine group 1.1 ± 1.5, placebo group 2.0 ± 2.0; AIMS, sarcosine group .1 ± .5, placebo group .4 ± 1.0; and Barnes Akathesia Scale (sarcosine group .5 ± 2.2, placebo group .0 ± .0).

Treatment-emergent adverse events in the placebo group included tremor ($n = 2$) and salivation ($n = 1$); the sarcosine group included tachycardia ($n = 2$). These systemic side effects were all short-lived and resolved spontaneously within days, not warranting medical treatment. They were likely coincidental observations.

The routine blood cell count and chemistry after 6 weeks of sarcosine or placebo treatment remained unchanged and were all within the normal ranges (data not shown).

Discussion

Our findings indicate that sarcosine, acting as an antagonist on the GlyT-1, can improve positive, negative, cognitive, and other psychiatric symptoms of schizophrenia. Since there is no other neurotransmission site at which sarcosine acts except the known GlyT-1 site, the effect of sarcosine is likely due to its action on the GlyT-1. Together with the positive findings of

D-serine (Tsai et al 1998a), glycine (Heresco-Levy et al 1999), and D-cycloserine treatments (van Berckel et al 1996; Goff et al 1999; Heresco-Levy et al 2002), all available evidence implies that hypo-NMDA neurotransmission in schizophrenia is relevant to the pathophysiology of schizophrenia and, importantly, that augmentation of the NMDA neurotransmission through the GlyT-1 or NMDA glycine site is a promising approach for the pharmacotherapy of schizophrenia.

Superior to the other NMDA glycine site agents, sarcosine extends its therapeutic effects beyond the core symptoms of schizophrenia. The PANSS-general subscale and the BPRS scores also improve; however, this will need to be confirmed in a parallel comparison study. In addition, our study indicates that sarcosine treatment can be beneficial for the patients receiving the atypical antipsychotic risperidone. Consistent with this, significant clinical effects were observed in patients receiving atypical antipsychotics clozapine and olanzapine by high-dose glycine treatment (Javitt et al 2001).

Sarcosine, like D-serine, improves both the positive and negative symptoms of schizophrenia, as well as the cognitive symptoms (Tsai et al 1998b). Sarcosine is demethylated to glycine by sarcosine dehydrogenase. Theoretically, therapeutic effects of sarcosine can be mediated by its metabolite, glycine. However, sarcosine dehydrogenase has the strongest expression in the liver and the expression in the brain is minimal (Bergeron et al 1998b). In addition, the dose of sarcosine is far less than that of the glycine (800 mg/kg/d) necessary to improve the symptoms of schizophrenia. Therefore, the therapeutic effect of sarcosine is unlikely due to its conversion to glycine by sarcosine dehydrogenase in the central nervous system (CNS). Nevertheless, since we cannot estimate the increase of brain sarcosine level after oral sarcosine administration, it is also possible that sarcosine converts to glycine preferentially in the CNS by a mechanism other than sarcosine dehydrogenase and acts as a prodrug of glycine.

Sarcosine and D-serine are superior to glycine (Heresco-Levy et al 1999) and D-cycloserine (van Berckel et al 1996; Goff et al 1999; Heresco-Levy et al 2002) in improving the positive symptoms of schizophrenia. Sarcosine has the similar magnitude of clinical efficacy in improving the negative symptoms of schizophrenia as D-serine (21%) (Tsai et al 1998a), glycine 5% (study 2, SANS) (Heresco-Levy et al 1996b) to 36% (Heresco-Levy et al 1999), and D-cycloserine (~20%) (van Berckel et al 1996; Goff et al 1999; Heresco-Levy et al 2002). But previous studies of glycine and D-cycloserine did not show effects on the positive symptoms. Nevertheless, a parallel comparison study is necessary to compare the efficacy of these NMDA-enhancing agents. The central bioavailability of sarcosine is unclear; however, the poor CNS bioavailability can explain the lack of effect of glycine on positive symptoms. D-cycloserine is a partial agonist. In addition, high concentration of D-cycloserine inhibits serine racemase (Cook et al 2002). Glycine and D-cycloserine may not be able to achieve the activation of NMDA neurotransmission required for the improvement of positive symptoms.

In addition to its primary role in neurotransmission, the NMDA receptor regulates synaptic plasticity, memory, and cognition (McDonald and Johnston 1990). Consistent with this, the patients on sarcosine improved significantly in their cognitive symptoms (Table 2). This cognition-enhancing effect through the NMDA-glycine site is also supported by the positive finding of our D-serine study (Tsai et al 1998a) and a glycine trial (Heresco-Levy et al 1996a). Further study of GlyT-1 and NMDA-glycine agents is required to explore their therapeutic potential on neurocognition.

Sarcosine does not worsen the side effects of other antipsychotics, which are mediated by dopamine type 2 (D2), serotonin type 2 (5-HT2), histamine, and muscarinic receptors. The extrapyramidal side effects, akathesia and dyskinetic movement, were not affected by sarcosine treatment. Similarly, D-serine, glycine, or D-cycloserine do not induce significant side effects (Goff et al 1999; Heresco-Levy et al 1999, 2002; Tsai et al 1998a, 1999). High doses of glycine were tolerated by the schizophrenic patients and no dropout was reported due to the side effects (Heresco-Levy et al 1996a, 1999). A vigorous review of systemic side effects reveals that sarcosine treatment at a dose of ~30 mg/kg/d is well-tolerated. The few side effects reported by the patients were minimal and coincidental and resolved spontaneously.

Sarcosine is a naturally occurring amino acid in humans. Toxicological properties of sarcosine have not been thoroughly investigated. However, sarcosine dehydrogenase is a mitochondrial matrix flavoenzyme expressed in the brain and liver to demethylate sarcosine (Eschenbrenner and Jorns 1999). The enzyme is defective in patients with sarcosinemia, a rare autosomal metabolic defect characterized by elevated levels of sarcosine in the blood and urine. Supporting the safety of using sarcosine as a long-term therapeutic agent of enhancing NMDA neurotransmission, sarcosinemia is probably benign (Eschenbrenner and Jorns 1999; Levy et al 1984) and the phenotype of sarcosine dehydrogenase mutant mice is unremarkable (Harding et al 1992).

Our study indicates that GlyT-1 antagonist sarcosine can improve the symptoms of schizophrenia with negligible side effects. Potentiation of NMDA neurotransmission by the antagonism of GlyT-1 represents a novel therapeutic approach that is worth further investigation.

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GT and H-YL contributed equally to this work.

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Short communication

A novel alanine-insensitive D-serine transporter in rat brain synaptosomal membranes

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Abstract

D-Serine is an endogenous modulator of brain *N*-methyl-D-aspartate receptors. This study investigates serine transport in brain synaptosomal fractions. Saturable, temperature-dependent uptake of both D- and L-[^{3}H]serine was observed. Alanine was only partially effective in blocking transport, arguing against involvement of system ASC. Inhibitors of the systems A, L and GLY were also ineffective. Saturation studies suggested a submillimolar K_m for both D- and L-serine. These data suggest the presence of a novel serine transport system in rodent synaptosomes. © 2002 Published by Elsevier Science B.V.

Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: Uptake and transporters

Keywords: D-Serine; Transporter; Synaptosome; NMDA receptor; Schizophrenia

Phencyclidine (PCP) and other antagonists of *N*-methyl-D-aspartate (NMDA)-type glutamate receptors induce psychotic symptoms that closely resemble schizophrenia, indicating that endogenous dysfunction or dysregulation of NMDA receptors may play a critical role in the pathogenesis of that disorder [6,12]. NMDA receptors are regulated by the endogenous amino acids glycine and D-serine, which bind to an NMDA-associated glycine binding site, leading to interest in the use of glycine-site agonists in the treatment of schizophrenia and in the study of endogenous processes regulating brain glycine and D-serine levels [11].

Both glycine and D-serine are present in extracellular fluid at low micromolar (μM) range concentrations [7,9]. In contrast, these agents bind to the NMDA/glycine site with affinity of approximately 500 nM [13], leading to the concern that the glycine site should be saturated under physiological conditions [3]. Saturation with glycine is apparently prevented by the action of glycine transporters (GLYT1) that are co-localized with NMDA receptors and maintain low, subsaturating concentrations in the immediate vicinity of the NMDA complex [19]. Cloned GLYT1 transporters show a K_m of ~100 μM for glycine. At present, little is known about mechanisms that prevent NMDA/glycine-site saturation with D-serine.

The transport system most associated with serine transport is system ASC, although transport may also occur though system L [17]. ASC systems, in general, have broad substrate specificity, making them unlikely candidates to serve as regulators of neurotransmitter levels. Expression studies also do not strongly support a role of system ASC. Several ASC-family transporters have been identified. ASCT1 transporters are expressed in brain, but appear to be insensitive to D-serine [1,18]. ASCT2 transporters show low μM affinity for D-serine. However, they appear not to be expressed in brain [20]. An Na^+-independent (system ASC) transporter has been cloned from both mouse [5] and human [15] and shown to have low μM-level affinity for D-serine. However, this transporter shows extremely broad substrate specificity (glycine, serine, threonine, cysteine, alanine), making it an unlikely candidate to regulate D-serine concentrations in vivo. Moreover, there is little endogenous Na^+-independent serine transport in brain, so the functional relevance of this transporter for regulating levels is unclear. Glioma cells accumulate D-serine with an apparent K_m of 2480 μM [10]. However, this transport process also exhibits extremely

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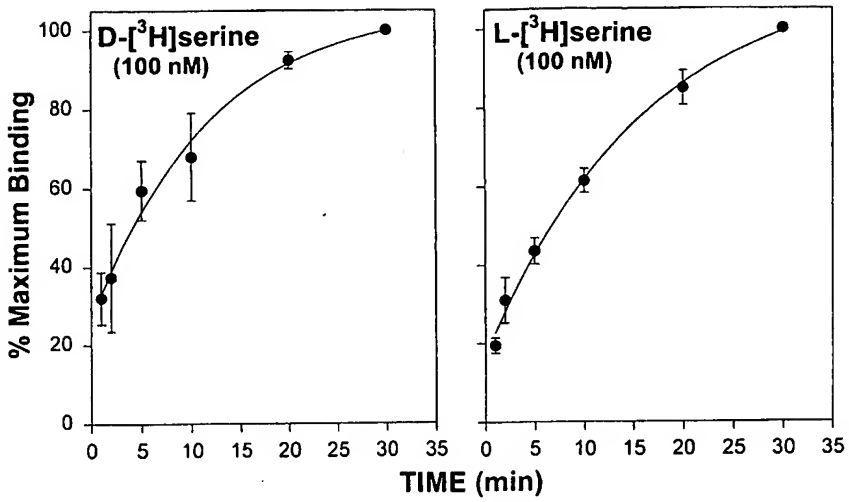

Fig. 1. Kinetics of D- and L-[^{3}H]serine uptake in rat P2 synaptosomal membranes. Data are mean±S.E.M. of three experiments, each performed in triplicate. Non-specific binding is determined in the presence of 30 mM D- or L-serine.

broad substrate specificity with much higher affinity for L- than D-[^{3}H]serine.

For the present study, assays were conducted using synaptosomal (P2) pellets prepared from rat brain. This fraction, which contains presynaptic terminals along with membrane from perisynaptic glial cells is most appropriate to studying transport mechanisms in the immediate vicinity of the synapse.

Synaptosomal (P2) preparations were prepared from cortex+hippocampus of Sprague–Dawley rats (200–250 g) using the method of Debler and Lajtha [4]. Membranes were suspended in Krebs solution (pH 7.4) containing 124 mM NaCl, 26 mM $NaHCO_3$, 10.5 mM glucose, 5 mM KCl, 1.3 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 2.4 mM $CaCl_2$, and were incubated at 37 °C in the presence of L- or D-[^{3}H]serine, as appropriate. Incubation was terminated by filtration under reduced pressure through Whatman GF/B filters, rinsing twice with 5 ml ice-cold buffer. Data in text represent mean±S.E.M. Statistical comparisons were performed using the two-tailed Student's *t* statistic.

For initial studies, uptake was measured over a 30 min period (Fig. 1). Uptake of L- and D-[^{3}H]serine was linear over the first 10 min with a tendency for plateau by 30 min. Uptake was not significantly affected by co-incubation with 10 mM 2-aminobicyclo (2,2,1)heptane-2 carboxylic acid (BCH; system L). Effects of the system ASC substrates alanine, cysteine and serine were evaluated at concentrations between 0.03 and 30 mM (Fig. 2). Com-

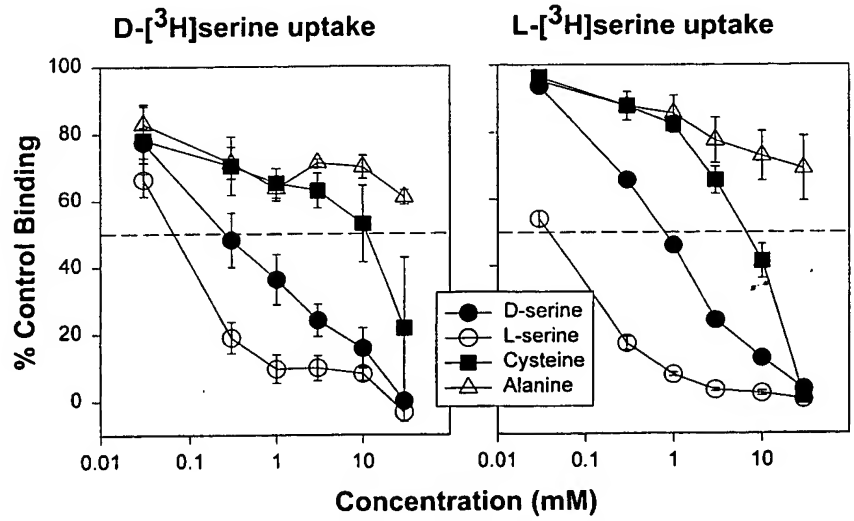

Fig. 2. Inhibition of D-[^{3}H]serine and L-[^{3}H]serine uptake (100 nM) by indicated amino acids. Data are mean±S.E.M. of three experiments, each performed in triplicate. The homogenate contained 1 mM concentrations of HA-966, L-*trans*-pyrollidine-2,4-dicarboxyllic acid (L-PDC), nipecotic acid, 2-aminobicyclo (2,2,1)heptane-2 carboxylic acid (BCH), methylaminoisobutyric acid (MeAiB) and 10 mM sarcosine, to inhibit binding to the NMDA-associated glycine site, and potential transport through glutamate, GABA, system L, system A, and system GLY, respectively [17].

plete inhibition of serine uptake was obtained with both L- and D-serine. L-Serine showed greater potency that D-serine in inducing inhibition. Inhibition was also obtained with cysteine, although potency of cysteine was significantly less than that of either L- or D-serine. In contrast, only partial inhibition was observed with L-alanine, even at doses as high as 30 mM.

In order to characterize kinetics of uptake, saturation studies were conducted following 5 min incubation with concentrations of L- and D-serine between 0.01 and 20 mM (Fig. 3). Saturation of both D- and L-serine binding was observed with half-maximal binding occurring between 0.5 and 2 mM. Michaelis–Menton constants (K_m) of 4.36±0.89 and 1.11±0.40 mM were obtained for L- and D-serine, respectively, by non-linear regression analysis, with corresponding V_{max} values of 96.3±10.6 and 60.5±4.8 pmol/mg protein/min, respectively. For both curve fits, r values were >0.95.

Although non-linear regression analysis did not obtain a two-site fit, Eadie–Hofstee plots showed apparent non-linearities for both D- and L-serine, with a higher affinity component being represented at concentrations below 0.1 mM. Linear regression analyses conducted using the initial slope of the Eadie–Scatchard plot revealed a high affinity component with affinity of 250 μM for D-serine and 301 μM for L-serine.

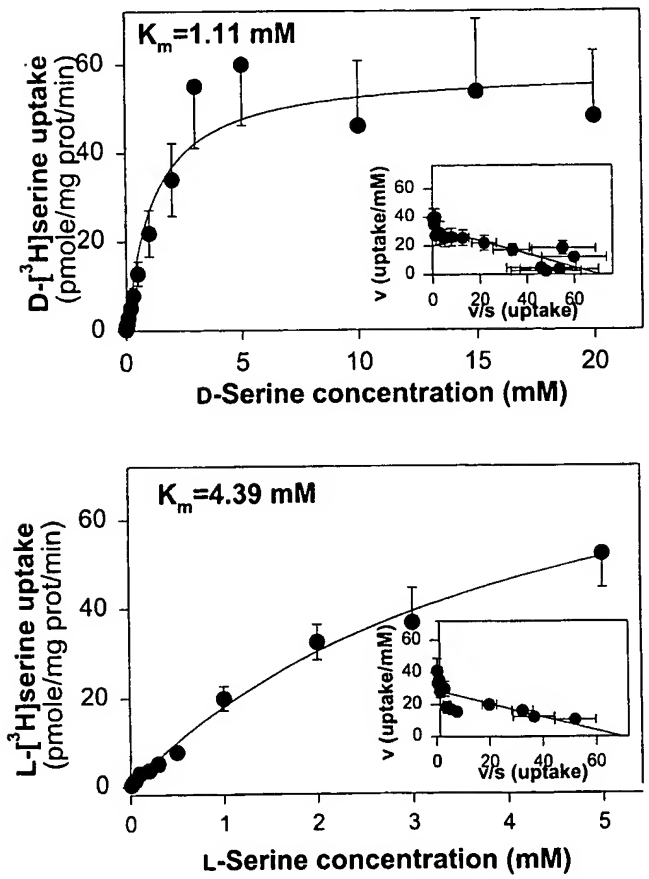

Fig. 3. Saturation analysis of D-[^{3}H]serine and L-[^{3}H]serine uptake. Data are mean±S.E.M. of 4–8 experiments each performed in triplicate. Non-specific binding was determined at 0 °C. Incubations were terminated following 5 min incubation. Assays were conducted in the presence of 30 mM L-alanine. K_m values were determined by non-linear regression to a single rectangular, three-parameter hyperbolic function using Sigmaplot 2000 (SPSS, Chicago, IL, USA). Inset, Eadie–Hofstee plot of D-[^{3}H]serine and L-[^{3}H]serine uptake.

Replacement of Na^+ by Li^+ in the Krebs buffer reduced specific D-[^{3}H]serine uptake following 30 min incubation to 47.1±4.3% of control (t=12.2, df=2, P<0.01). Replacement of Cl^- by acetate reduced uptake to 68.8±6.0% of control (t=5.2, df=2, P<0.05). Replacement of both Na^+ and Cl^- led to a reduction to 41.0±2.6% of control (t=23.3, df=2, P<0.002).

D-Serine is an important neuromodulator in mammalian brain that may serve as the endogenous ligand for the NMDA-associated glycine binding site [8,14]. Brain serine transport, in general, is postulated to be mediated by system ASC transporters, which transport alanine as well as serine. This study demonstrates that, in synaptosomes, the majority of uptake occurs via an alanine-insensitive, presumably non-system ASC, transporter. Uptake by this system was observed in the presence of the NMDA/glycine-site antagonist HA-966 and of canonical inhibitors of glutamate and GABA transport and inhibitors of system A, L and ASC (Fig. 2). This finding, therefore, indicates the presence of a novel, previously undescribed amino acid transporter that may play a physiological role in modulating NMDA receptor-mediated neurotransmission. Transport through this system was nevertheless Na^+/Cl^- sensitive.

An issue with regard to the present system is whether it is of sufficiently high affinity to maintain submicromolar concentrations of D-serine in the vicinity of NMDA receptors. It has been estimated that glycine transporters are capable of maintaining glycine concentrations as low as 100 nM [16]. Several factors are important in determining transporter-maintained extracellular amino acid levels. These include (1) transporter affinity, (2) the degree of electrogenic coupling, and (3) the intracellular concentration of the agent to be transported. In general, it has been estimated that transporters can maintain 10,000–1,000,000-fold gradients, depending upon the degree of electrogenic coupling [2]. Whether or not the affinity of the D-serine transporter described in this study is sufficient to maintain submicromolar extracellular D-serine levels will depend both on the intracellular concentration of D-serine within brain elements and on the number of Na^+ ions countertransported with D-serine. These values have not been determined.

Further, the critical issue for functional regulation of D-serine levels may not be the absolute affinity of the transporter, but rather its specificity for amino acids other than serine. The transporter detected here is the only serine transporter thus far described that has limited affinity for other neutral amino acids including cysteine and alanine, and the only one identified specifically in the P2 fraction.

Thus, it is a reasonable candidate to serve as an endogenous regulator of local synaptic D-serine concentration in brain.

Because linearity of uptake in this study was only determined for a low concentration (100 nM) of D- and L-serine, it is possible that uptake rate was underestimated at higher concentrations. This would lead to an overestimation of K_m values. Further, it is likely that multiple transport systems contributed simultaneously to the observed uptake, leading to an apparent higher affinity of L-serine relative to D-serine in the competition assays vs. uptake studies. Detailed characterization of this transporter will likely require expression cloning in oocytes or other appropriate expression system. Nevertheless, this study suggests that transport systems for serine in synaptosomes may differ from those in other brain compartments.

Acknowledgements

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Inhibition of striatal dopamine release by glycine and glycyldodecylamide

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ABSTRACT: Phencyclidine (PCP) and other N-methyl-D-aspartate (NMDA) antagonists induce schizophrenia-like symptoms in humans. In rodents, PCP induces a syndrome of stereotypies and hyperactivity that is accompanied by stimulation of striatal dopamine release. Glycine and other NMDA agonists reverse PCP-induced behaviors in rodents and ameliorate PCP psychosis-like symptoms of schizophrenia in clinical trials. Glycine levels *in vivo* are regulated by the actions of glycine (GLYT1) transporters. The present study investigates effects of glycine and the prototypic glycine transport inhibitor glycyldodecylamide (GDA) on striatal dopamine release *in vitro* using a mouse striatal assay. Glycine and GDA significantly inhibit NMDA-induced striatal dopamine release, consistent with their ability to enhance local striatal inhibitory neurotransmission *in vitro* and to reverse PCP-induced hyperactivity *in vivo*. © 2000 Elsevier Science Inc.

KEY WORDS: NMDA receptors, Phencyclidine, Schizophrenia, Mouse.

INTRODUCTION

Schizophrenia is a major mental disorder affecting 0.5–1.0% of the population worldwide. Traditional models of schizophrenia postulate that abnormalities of brain dopaminergic function may be etiologic in schizophrenia. In contrast, several more recent models have focused on dysfunction of brain glutamatergic systems. Glutamatergic theories of schizophrenia are based primarily upon the observation that phencyclidine (PCP), ketamine and other N-methyl-D-aspartate (NMDA) antagonists induce schizophrenia-like symptoms in healthy volunteers and exacerbate symptoms in remitted schizophrenic patients. Such models are also supported by animal studies demonstrating that noncompetitive NMDA antagonists induce schizophrenia-like disturbances in social and cognitive behaviors (for review, see [4,11,12]).

NMDA receptors are activated not only by glutamate but also by glycine, which binds to an allosteric modulatory site on the NMDA complex. Glycine-site agonists, including glycine and D-serine (full agonists) and D-cycloserine (a partial agonist) have been shown to be clinically beneficial in schizophrenia [5,7,24], supporting NMDA-based models. In rodents, PCP induces a characteristic syndrome of stereotypies and locomotor hyperactivity that is associated with increased striatal dopamine release [8,12]. PCP-induced hyperactivity is inhibited by glycine and other glycine-site agonists [9,10,17], consistent with the ability of glycine to inhibit PCP-induced striatal dopamine release *in vivo* [8].

Mechanisms by which glycine inhibits PCP-induced hyperactivity and striatal dopamine release are presently unknown. NMDA receptors are known to exert multiphasic effects on striatal dopamine release *in vivo* [25]. NMDA receptors on presynaptic dopaminergic terminals stimulate dopamine release. In contrast, NMDA receptors on intrinsic γ-aminobutyric acid (GABA)ergic neurons stimulate GABA release leading to local dopamine-release inhibition. Glycine potentiates NMDA-stimulated dopamine release from isolated striatal synaptosomes and reverses glycine antagonist-induced inhibition of dopamine release from striatal slices [13,16,19]. Whether or not glycine also potentiates inhibitory neurotransmission within striatum, however, is unknown. The present study investigates effects of glycine on NMDA-stimulated striatal dopamine release in isolated striatum in order to evaluate potential excitatory and inhibitory effects.

Glycine levels in brain are regulated by glycine (GLYT1) transporters which are co-localized with NMDA receptors [22] and which may play a physiological role in regulation of NMDA receptor-mediated neurotransmission [9,10,23]. The present study also evaluates excitatory and inhibitory effects of glycyldodecylamide (GDA), a prototypic glycine transport antagonist, that induces glycine-like inhibition of PCP-induced locomotor hyperactivity *in vivo* [8,9].

MATERIALS AND METHODS

Mice were decapitated and the striatal tissue (approx. 8–10 mg) was dissected out and incubated for 60 min in Mg^{2+}-containing 0.5 ml of Krebs bicarbonate buffer containing 1.25 μCi [^{3}H]dopamine (New England Nuclear, Boston, MA, USA). The reaction mixture was continuously gassed with an O_2/CO_2 mixture (95%/5%). After prelabeling, tissue was transferred to superfusion chambers (12 × 0.3 ml chambers, Brandel Superfusion 1200, MD) and pre-perfused at a rate of 0.4 ml/min (Figs. 1–3) or 0.8 (Fig. 4) ml/min for 60 min in the above buffer minus Mg^{2+}. Effluent was discarded during this period and thereafter 4-min (Figs. 1–3) or 3-min (Fig. 4) fractions were collected for an additional 45–60 min. Blocking drugs were added during fraction 3 and maintained until the end of the experiment. Enhanced release was induced by

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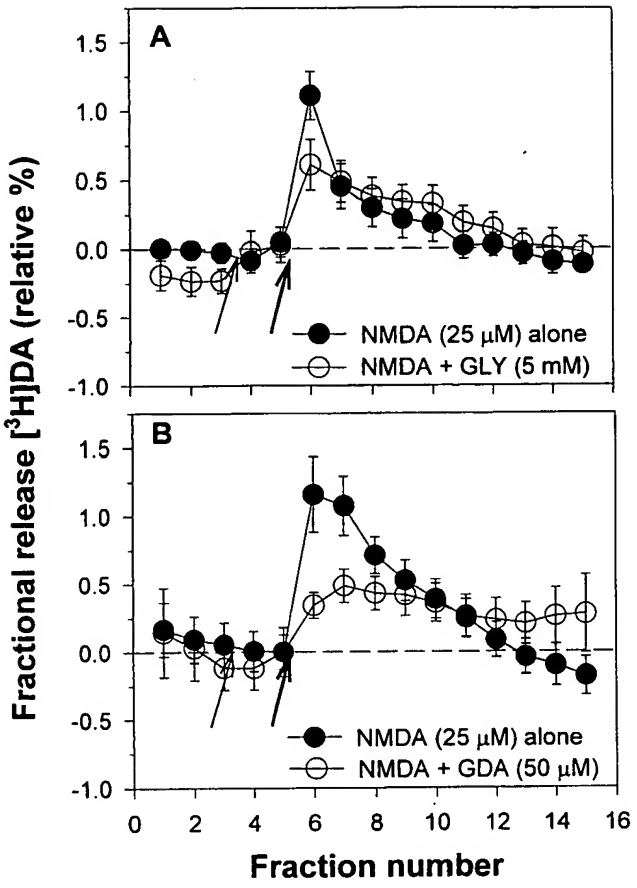

FIG. 1. Representative experiments showing effects of (A) glycine (GLY) and (B) glycyldodecylamide (GDA) on N-methyl-D-aspartate (NMDA)-stimulated dopamine ([^{3}H]DA) release. Data (± SEM) are from 3 min fractions. GLY, GDA or vehicle were added following fraction 3 (thin line). NMDA was added after fraction 5 (thick line). Release was defined as summed area under the release curve between fractions 5 and 12 relative to the fraction 4/5 baseline.

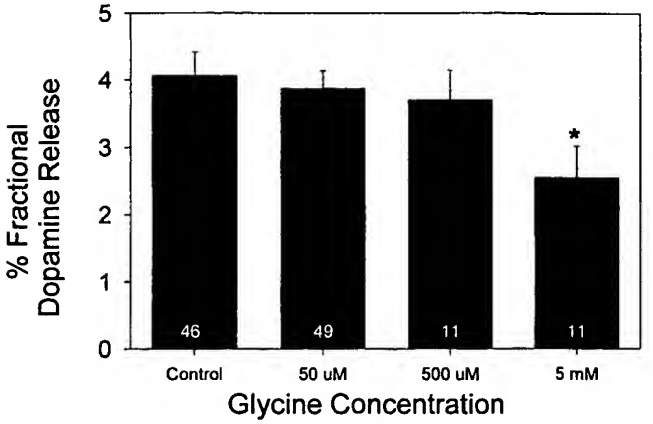

FIG. 2. Bar chart showing effect of glycine on N-methyl-D-aspartate-stimulated dopamine release. Number of experiments are shown in inset. *$p < .05$; **$p < .01$ vs. Control.

addition of the drugs during fraction 5 (2-min exposure). Fractional release was defined as radioactivity content of each fraction divided by the amount of radioactivity remaining in the tissue at the time the fraction was collected. NMDA-induced release was defined as the area under the fractional release curve for 1/2 h following NMDA exposure (i.e., between fractions 5 and 12). Following the perfusion period (fraction 15), the tissue was removed from the perfusion chamber, suspended in 500 μl of 0.2 N NaOH and sonicated. An aliquot (100 μl) was assayed for tissue radioactivity. To determine radioactivity released from the tissue, the perfusate (1.6 ml) was mixed with 3 ml Liquiscint scintillation fluid (National Diagnostics, Atlanta, GA, USA) and counted in a Packard Scintillation Counter (Model 1500).

RESULTS

An initial series of experiments investigated effects of increasing concentrations of glycine between 50 μM and 5 mM. Glycine was added at fraction 3, followed by NMDA at fraction 5 (Fig. 1). Glycine itself led to small but significant enhancement of basal dopamine release ($t = 2.68$, $p = .02$) as reflected in lesser release in fractions 1 and 2 (pre-glycine) than in fractions 4 and 5 (post-glycine) in the glycine condition (Fig. 1A). However, relative to the fraction 4/5 baseline, NMDA-stimulated release was significantly lower in the presence than absence of 5 mM glycine ($t = 2.00$, $p = .05$) (Fig. 2).

A second series of experiments evaluated effects of the glycine transport inhibitor GDA. GDA had no significant effect on basal dopamine release (Fig. 1B). However, incubation in the presence of increasing concentrations of GDA between 10 μM and 1 mM led to a significant dose-dependent decrease in NMDA-stimulated dopamine release, with significant reductions relative to control at both 50 ($t = 3.62$, $p < .001$) and 100 (M ($t = 2.58$, $p < .02$) concentrations (Fig. 3). The degree of reduction in release in the presence of 50 μM GDA was similar to that observed in the presence of 5 mM glycine.

In order to verify that effects of GDA were mediated at NMDA/glycine receptors, studies were conducted in the absence and presence of the selective glycine-site antagonist L689,560 (Fig. 3). Incubation in the presence of L689,560 (1 nM) alone did not significantly inhibit dopamine release. Nevertheless, in the presence of L689,560 (1 nM), effects of GDA (50 μM) were no longer significant. A 2 × 2 analysis of variance evaluated interactive effects of L689,560 and GDA on NMDA-induced dopamine release. A significant GDA × L689,560 interaction was observed ($F = 5.0$, $df = 1/135$, $p = .03$), reflecting significant reversal of GDA-induced inhibition by L689,560. A higher dose of L689,560 (100 nM) significantly inhibited dopamine release ($t = 4.22$, $p < .001$). This inhibition was partially reversed by 10 uM GDA ($t = 1.86$, $p = .08$), although release in the presence of combined L689,560 (1 μM) and GDA (10 μM) remained significantly below control levels ($t = 3.15$, $p = .003$).

A final series of experiments investigated the ability of L689,560 to reverse effects of high-dose glycine (Fig. 4). Studies were performed in the presence of 5 and 10 mM concentrations of glycine. As in the initial series of experiments, incubation in the presence of 5 mM glycine led to a decrease in dopamine release to 60% of control levels. Incubation in the presence of 10 mM led to a further reduction to 35% of control levels ($t = 5.31$, $p < .001$). Pre-incubation with L689,560 (10 nM) partially prevented the effect of high-dose glycine ($t = 2.80$, $p = .009$), although release remained lower than under control conditions ($t = 3.32$, $p = .002$).

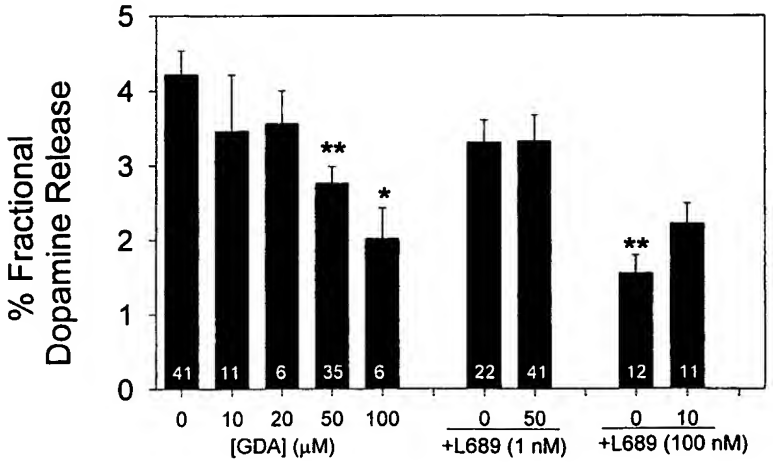

FIG. 3. Bar chart showing effects of glycyldodecylamide (GDA) alone or in combination with the glycine site inhibitor L689,560 (L689) on N-methyl-D-aspartate-stimulated dopamine ([^{3}H]DA) release. *$p < .05$; **$p < .01$ vs. Control.

DISCUSSION

Elevated striatal dopamine levels are associated with both PCP-induced hyperactivity and clinical symptoms of schizophrenia [11,12]. The major finding of the present study is that glycine, an NMDA receptor co-agonist, significantly inhibited NMDA-induced striatal dopamine release when applied at doses in the low millimolar range, consistent with its ability to reverse PCP-induced hyperactivity and ameliorate negative symptoms of schizophrenia. GDA, a protoypic glycine transport antagonist, induced a similar effect, but was active at micromolar concentrations. The actions of both glycine and GDA were inhibited by L689,560, supporting the concept that glycine- and GDA-induced inhibition of striatal dopamine release is mediated at an NMDA-associated glycine binding site.

The present results are consistent with several recent studies demonstrating that NMDA receptors within striatum *in vivo* act primarily to inhibit dopamine release via stimulation of inhibitory interneurons [25]. These interneurons are also stimulated by dopamine, acting at D1 receptors [6], and so form part of the normal local feedback loop that regulates striatal dopamine release [20].

Prior studies utilizing striatal slices have not demonstrated inhibitory effects of glycine [14,16,19]. However, the concentrations of glycine used in those studies were significantly lower than the doses used in the present study. Concentrations used in the present study are similar to those that are required to potentiate NMDA activation in slices obtained from rat hypoglossal nucleus [1], and to overcome the buffering capacity of GLYT1 transporters expressed *in vitro* [21]. Concentrations are also similar to those used to stimulate cortical NMDA receptor-mediated neurotransmission *in vivo* [18]. In the absence of NMDA, glycine significantly potentiated basal dopamine release, suggesting that glycine may also potentiate release from presynaptic dopamine terminals. However, at high glycine concentrations, the inhibitory effects predominated due perhaps to lower endogenous glycine concentrations in the vicinity of post-synaptic vs. pre-synaptic NMDA receptors.

GDA, a prototypic glycine transport antagonist, induced neurochemical effects similar to those of glycine itself. In rodents, GDA reverses PCP-induced hyperactivity with potency approximately 10-fold greater than that of glycine itself. In rat synaptosomes, GDA inhibits glycine transport at concentrations in the high micromolar range [9]. The potency of GDA thus corresponds to its potency for inhibiting GLYT1-mediated transport. GLYT1 inhibitors have previously been shown to potentiate NMDA receptor-mediated neurotransmission in rat hippocampal slices [2]. This is the first study to demonstrate GLYT1 inhibitor-induced inhibition of striatal dopamine release. Several recent studies have demonstrated that schizophrenia is associated with hyper-reactivity of striatal dopamine release [3,15]. To the extent that such studies are correct, the ability of glycinergic agents to inhibit subcortical dopamine release would be expected to be therapeutically beneficial in schizophrenia.

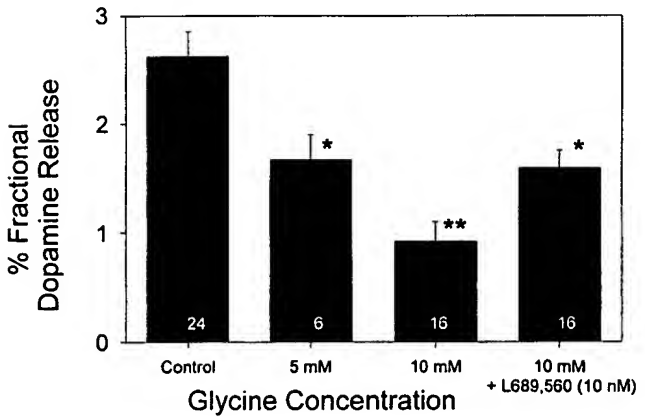

FIG. 4. Bar chart showing effects of L689,560 on glycine-induced inhibition of N-methyl-D-aspartate-stimulated dopamine. *$p < .05$; **$p < .01$ vs. Control.

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Modulation of Striatal Dopamine Release by Glycine Transport Inhibitors

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Traditional models of schizophrenia have focused primarily upon dopaminergic (DA) dysregulation. In contrast, more recent models focus on dysfunction of glutamatergic systems, acting particularly through *N*-methyl-D-aspartate (NMDA) receptors. NMDA receptors in brain are regulated by glycine, acting via a strychnine-insensitive regulatory site, and by glycine (GlyT1) transporters that maintain low glycine levels in the immediate vicinity of the NMDA receptor complex. The present study investigates the role of NMDA receptors in the modulation of striatal dopamine release *in vitro*, and of glycine transport inhibitors (GTIs) as potential psychotherapeutic agents in schizophrenia. In striatum, NMDA receptors exert dual excitatory/inhibitory effects, with inhibition reflecting activity of local GABAergic feedback regulation. We have previously demonstrated effectiveness of glycine in regulating [^{3}H]DA release both *in vivo* and *in vitro*, consistent with its beneficial clinical effects. In the present study, similar effects were observed for the high-affinity GTI (+)*N*[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy-)propyl]sarcosine (NFPS), and for a range of high-affinity GTIs with appropriate rank order of potency. In addition, (+)NFPS significantly stimulated NMDA-induced [^{3}H]GABA release. Effects, of GTIs, were blocked by the glycine-site antagonists L689,560 and HA-966, and the $GABA_B$ antagonists phaclofen and CGP 52432, confirming the roles of both the NMDA-associated glycine-site and presynaptic $GABA_B$ receptors in NMDA receptor-mediated regulation of striatal DA release *in vitro*. Endogenous DA hyperactivity is associated with prominent positive symptoms in schizophrenia. The present results are consistent with recent clinical studies showing significant effectiveness of glycine-site agonists and GTIs in reduction of persistent positive, as well as negative, symptoms in schizophrenia.

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Keywords: glutamate; NMDA receptor; amphetamine; phencyclidine; schizophrenia

INTRODUCTION

Schizophrenia is a severe brain disease that affects up to 1% of the world's population. Traditional models of schizophrenia emphasize the importance of dopaminergic (DA) dysregulation (eg, Davis *et al*, 1991; Moore *et al*, 1999), particularly in the etiology of positive symptoms. More recent models, in contrast, emphasize the potential role of *N*-methyl-D-aspartate (NMDA) receptor dysfunction as a basis for both persistent negative and cognitive symptoms, and DA dysregulation in schizophrenia (Javitt, 1987, 2004; Carlsson and Carlsson, 1990; Javitt and Zukin, 1991; Coyle, 1996; Hirsch *et al*, 1997; Abi-Saab *et al*, 1998; Olney *et al*, 1999; Moghaddam and Jackson, 2003). This reconceptualization of schizophrenia has led to efforts both to develop more effective NMDA-stimulating agents, and to define critical sites of interaction between dopamine and NMDA systems.

In schizophrenia, DA dysregulation, particularly in striatum, has been confirmed through *in vivo* neuroimaging (Abi-Dargham *et al*, 1998; Breier *et al*, 1997; Laruelle, 1998; Laruelle *et al*, 1996). NMDA receptors are present in high density in striatum, and are localized on both presynaptic DA terminals and on GABA interneurons where they serve to inhibit presynaptic DA release through local feedback regulation (Wu *et al*, 2000). NMDA receptor antagonists, such as phencyclidine (PCP) or ketamine, induce schizophrenia-like DA dysregulation in both animals (Balla *et al*, 2001, 2003; Miller and Abercrombie, 1996) and normal human volunteers (Kegeles *et al*, 2000). Further, mice with mutations of the NMDA ε1 subunit show both biochemical and behavioral evidence of DA dysregulation (Ballard *et al*, 2002; Miyamoto *et al*, 2001). Dysfunction or blockade of NMDA receptors particularly on GABA interneurons may,

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therefore, produce DA hyperactivity similar to that observed in schizophrenia.

NMDA receptors in brain are regulated both by glutamate, which serves as the primary agonist, and glycine, which binds to an allosteric modulatory site (Dingledine *et al*, 1990; Reynolds and Miller, 1990). Glycine levels in brain, in turn, are regulated by glycine transporters including the glycine type I (GlyT1) transporter. GLYT1 transporters are colocalized with NMDA receptors and serve to 'protect' the NMDA site from high circulating levels of glycine (Lopez-Corcuera *et al*, 2001; Smith *et al*, 1992). One recent approach to NMDA potentiation, therefore, has been the development of glycine transport inhibitors (GTIs), which prevent glycine removal from the synaptic cleft thereby increasing occupancy of the NMDA/glycine-binding site (Javitt and Frusciante, 1997; Javitt *et al*, 1997; Supplisson and Bergman, 1997).

In initial studies, the prototypic glycine transport inhibitor glycyldodecylamide (GDA) significantly inhibited PCP-induced hyperactivity in rodents (Javitt *et al*, 1999; Toth *et al*, 1986) and reversed NMDA-induced DA release from rat striatal slices (Javitt *et al*, 2000). More recently, high-affinity glycine transporter inhibitors such as *N*[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)propyl]sarcosine (NFPS, ALX5407) (Atkinson *et al*, 2001; Herdon *et al*, 2001) have been shown to potentiate NMDA receptor-mediated neurotransmission both *in vitro* (Bergeron *et al*, 1998) and *in vivo* (Chen *et al*, 2003); to normalize prepulse inhibition (Kinney *et al*, 2003; Le Pen *et al*, 2003); and to reverse PCP-induced DA dysregulation *in vivo* (Javitt *et al*, 2003). However, mechanisms underlying DA reregulation produced by NMDA agonists, including both glycine and newly developed glycine transport inhibitors, remain to be determined. In the present study, an *in vitro* striatal release assay, shown previously to be sensitive to effects of glycine and the prototype glycine transport inhibitor GDA (Javitt *et al*, 2000), was used to evaluate effects of newly developed glycine transport inhibitors on striatal DA and GABA release, and to investigate mechanisms underlying NMDA/DA/GABA interactions.

METHODS

Mice were decapitated and the striatal tissue (approximately 8–10 mg) was dissected out and incubated for 60 min in Mg^{2+}-containing 0.5 ml of Krebs bicarbonate buffer containing 1.25–3.0 μCi [^{3}H]dopamine (New England Nuclear, Boston, MA) and, where applicable, 1.2 μCi [^{14}C]GABA. For striatal dissections, the mouse brain was partially dissected in half and the cortex carefully folded outwards with a small spatula to expose the striatum from each hemisphere. The area of the striatum was visible from the surrounding region by their striated texture. The spatula was used to undercut the region slightly below the surface and to remove the striatal tissue; approximately 15 mg for both left and right striatum. For experiments involving GABA, amino-oxyacetic acid (0.1 mM) was added to the incubation buffer. The reaction mixture was continuously gassed with an O_2/CO_2 mixture (95%/5%). After prelabeling, tissue was transferred to superfusion chambers (12 × 0.3 ml chambers, Brandel Superfusion 1200, MD) and preperfused at a rate of 0.8 ml/min for 60 min in the above buffer minus Mg^{2+}. Effluent was discarded during this period and thereafter 3 or 4 min fractions were collected for an additional 45–60 min, depending on the experiment. Blocking drugs were added before indicated fractions and maintained until the end of the experiment.

Enhanced release was induced by addition of NMDA, at indicated doses, during fraction 5 (2 min exposure). Fractional release was defined as radioactivity content of each fraction divided by the amount of radioactivity remaining in the tissue at the time the fraction was collected. For studies using 25 μM NMDA, NMDA-induced release was defined as the area under the fractional release curve for 24 min following NMDA exposure (fractions 6–11), relative to surrounding fractions. For studies using 300 μM NMDA, NMDA-induced release was defined as the summed release during the post-NMDA exposure period (fractions 6–15), relative to baseline.

Following the perfusion period (fraction 15), the tissue was removed from the perfusion chamber along with the remaining perfusion buffer, sonicated, and assayed for tissue radioactivity. To determine radioactivity released from the tissue, the perfusate (2.4 ml) was mixed with 3 ml Liquiscint scintillation fluid (National Diagnostics, Atlanta, Georgia) and counted in a Packard Scintillation Counter (Model 1500).

Effects of GTIs on synaptosomal glycine transport were determined as previously described (Javitt and Frusciante, 1997). Assays were performed using a concentration of 1 μM of prospective GTIs.

Data in text represent mean ± SD. Two-tailed statistics with preset alpha level for significance of $p < 0.05$ are used throughout.

RESULTS

The goal of this study was to investigate effects of newly developed GTIs on NMDA-stimulated DA release in rodent striatum. Initial studies were performed with enantiomers of ALX5311, a high-potency GTI structurally related to NFPS. Subsequent studies were performed with the resolved enantiomers (+) and (−)NFPS. L689,560, a high-affinity NMDA/glycine-site antagonist (Stauch Slusher *et al*, 1994) and HA-966 (Javitt *et al*, 2000), a partial agonist, were used to evaluate involvement of NMDA receptors in GTI-induced effects. The $GABA_B$ agonist baclofen, and antagonists phaclofen (Kerr *et al*, 1987) and CGP52432 (Teoh *et al*, 1996) were used to evaluate potential $GABA_B$ receptor involvement.

Effect of Glycine Transport Inhibitors on NMDA-Induced Dopamine Release

An initial set of studies was performed in Balb/c mice. In the absence of added ligands, brief exposure of 25 μM NMDA induced a robust increase in dopamine efflux that peaked immediately following NMDA exposure and decayed over the ensuing 28 min (seven fractions). Initial studies were performed using 1 μM ALX5311, a potent, selective GTI (Figure 1). ALX5311 inhibited NMDA-induced dopamine release in a dose-dependent fashion ($F = 4.82$, $df = 3/126$,

$p = 0.003$) with highly significant inhibition being observed at a dose of 1 μM ($t = 3.75$, df = 116, $p < 0.0001$) (Figure 2a). Further, the (+) enantiomer exerted significantly greater inhibition of release than the (−) enantiomer ($t = 3.03$, df = 19, $p = 0.014$).

In order to determine role of the NMDA-associated glycine site in this effect, studies were repeated in the presence of the selective glycine-site antagonist L689,560 and the partial antagonist HA-966 (Figure 2b). NMDA-induced DA release was significantly inhibited by L689,560 itself in a dose-dependent fashion, with significant inhibition being observed at a dose of 100 nM ($t = 3.94$, df = 103, $p < 0.0001$). In the presence of L689,560, no significant effects of ALX5311 were observed ($t = 1.14$, df = 36, $p = 0.3$). When data were entered into a 2 × 2 ANOVA, a significant L689,560 × ALX5311 interaction emerged (F = 7.68, df = 1/152, $p = 0.006$), reflecting the significant effects of ALX5311 in the absence but not the presence of L689,560. HA-966 alone did not significantly inhibit NMDA-induced DA release at a concentration of 10 μM. Nevertheless, in the presence of HA-966, no significant further effect of ALX5311 was observed.

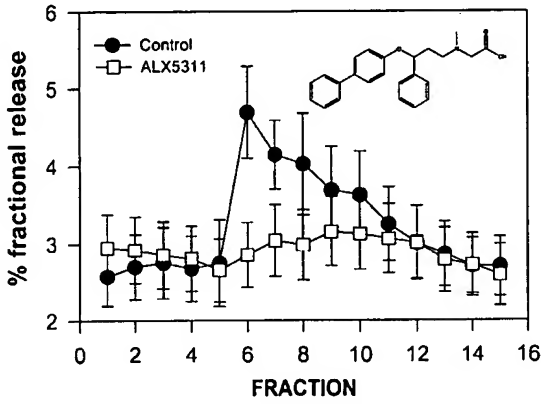

Figure 1 Fractional NMDA-stimulated [^{3}H]DA release in the absence and presence of the high-affinity glycine transport inhibitor ALX5311 (1 μM). Structure of ALX5311 is shown in inset. Fractions were collected every 4 min. Data are mean ± SEM. *$p < 0.05$ *vs* control; **$p < 0.01$ *vs* control.

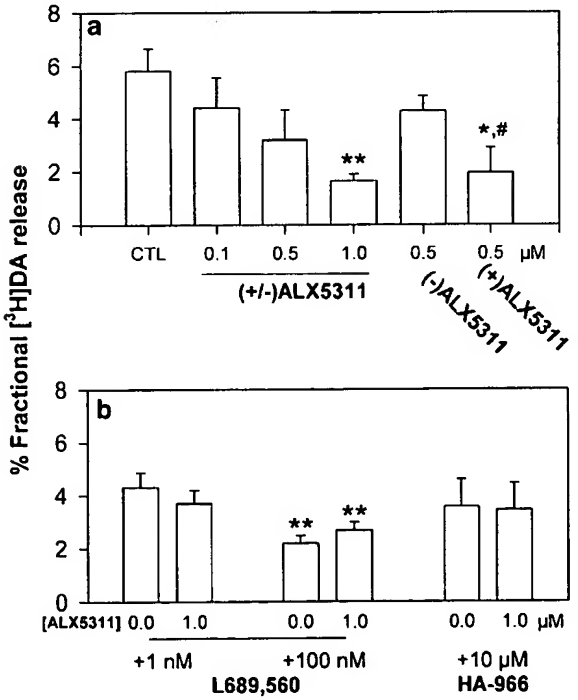

Figure 2 Summed NMDA-stimulated [^{3}H]DA release in the absence and presence of (+) and (−) ALX5311 (a) and in the additional presence of the NMDA/glycine-site antagonists L689,560 and HA-966 (b). *$p < 0.01$ *vs* control, **$p < 0.001$ *vs* control; #$p < 0.05$ *vs* (−)ALX5311.

In order to verify the effects of ALX5311, experiments were repeated with the subsequently developed, selective GLYT1 inhibitor (+) NFPS. As with ALX5311, the (+) isomer shows greater affinity for the GLYT1 transporter than the (−) isomer (Kinney *et al*, 2003; Mallorga *et al*, 2003), permitting differential effectiveness studies to be performed. (+)NFPS was significantly more effective than (−)NFPS ($t = 2.32$, df = 37, $p = 0.05$), which was without significant effect (Figure 3). Across this series of NFPS-type drugs, the degree of inhibition of [^{3}H]DA release correlated significantly with the degree of inhibition of synaptosomal glycine transport ($p = 0.01$).

Owing to the change in institutional breeding policies, a second set of studies with (+)NFPS was performed in C57BL/6 mice to verify stability of effect across strains (Figure 4). In order to optimize conditions, studies were performed in both the absence and presence of added glycine (100 μM). In tissue from these mice, (+)NFPS also induced significant, dose-dependent inhibition of NMDA-stimulated [^{3}H]DA release (F = 5.0, df = 2/334, $p = 0.007$). The effect of glycine was significant only at trend level (F = 3.3, df = 2/334, $p = 0.07$). No significant glycine × (+)NFPS interaction was observed (F = 1.5, df = 2/334, $p = 0.2$).

Effect of GTIs on [^{3}H]GABA Release

One hypothetical mechanism by which GTIs might induce their effects is by modulation of GABAergic feedback regulation. In order to investigate effects of GTIs on striatal

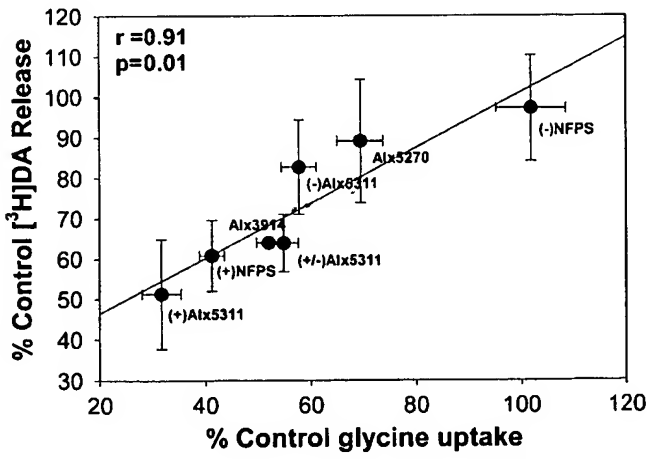

Figure 3 Correlation between degree of inhibition of NMDA-stimulated DA release in mouse striatal release assay at concentrations of 1 μM and degree of inhibition of [^{3}H]glycine uptake in a rat synaptosomal [^{3}H]glycine uptake assay.

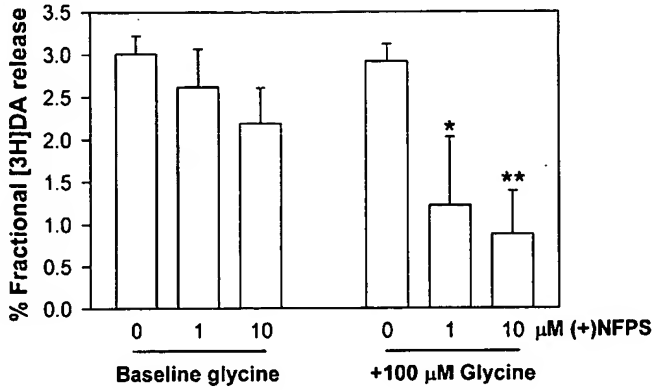

Figure 4 Effect of the high-affinity GTI (+)NFPS on (a) basal, and (b) NMDA-stimulated [^{3}H] release in the absence and presence of added glycine (100 μM). Fractions were collected every 3 min. *$p<0.01$; **$p<0.001$ *vs* control.

GABAergic function, striata were dually preincubated with [^{14}C]GABA and [^{3}H]DA. For these experiments, a higher dose (300 μM) of NMDA was used to increase release levels. (+)NFPS was employed at a dose of 10 μM. In this experiment, compounds were added at fraction 1 to permit greater evaluation of effects on basal release, as well as on NMDA-stimulated DA release. L689,560 (100 nM) was used to evaluate NMDA/glycine-site dependence of (+)NFPS effects.

As in prior studies, (+)NFPS had no effect on basal [^{3}H]DA release in either the absence ($t=.1$, df = 100, $p=0.9$) or presence ($t=1.35$, df = 52, $p=0.2$) of added L689,560 (Figure 5a). In contrast, L689,560 (100 nM) significantly enhanced basal release of [^{3}H]DA ($t=3.07$, df = 94, $p=0.003$). An ANOVA revealed a significant main effect of L689,560 ($F=9.4$, df = 1/152, $p=0.003$), on basal DA release, but no significant main effect of (+)NFPS ($F=2.1$, df = 1/152, $p=0.1$) or (+)NFPS × L689,560 interaction ($F=1.9$, df = 1/152, $p=0.2$).

As in prior experiments, (+)NFPS significantly inhibited NMDA-induced release [^{3}H]DA in an L689,560-dependent fashion (Figure 5b). In the absence of added L689,560, significant inhibition by (+)NFPS was observed ($t=3.61$, df = 100, $p<0.001$). L689,560 by itself also inhibited NMDA-induced release ($t=4.68$, df = 94, $p<0.001$). However, in the presence of L689,560, (+)NFPS had no significant further inhibitory effect ($t=0.4$, df = 52, $p=0.7$). ANOVA, therefore, revealed a significant main inhibitory effect of L689,560 ($F=14.6$, df = 1/152, $p<0.001$). Although (+)NFPS had no significant effect on its own ($F=3.1$, df = 1/152, $p=0.08$), the (+)NFPS × L689,560 interaction effect was significant ($F=5.71$, df = 1/152, $p=0.02$), reflecting the differential effect in the presence *vs* absence of added glycine-site antagonist and confirming prior observations.

Predicted effects of (+)NFPS were also observed on GABA release. Thus, both basal ($F=14.8$, df = 1/224, $p<0.001$) (Figure 5c) and NMDA-stimulated ($F=9.97$, df = 1/224, $p=0.002$) (Figure 5d) release of [^{14}C]GABA was significantly higher in the presence of (+)NFPS than in its absence. However, L689,560 had a more complex effect, in that it significantly increased basal release ($F=11.1$, df = 1/224, $p=0.001$), but significantly inhibited NMDA-induced release ($F=4.74$, df = 1/224, $p=0.03$). No significant (+)NFPS X L689,560 interaction were observed for either basal ($F=.5$, df = 1/224, $p=0.5$) or NMDA-stimulated ($F=1.26$, df = 1/224, $p=0.3$) release. These findings demonstrate the predicted ability of GTIs to enhance NMDA-regulated GABA release in striatum, although it suggest the presence of multiple sites of action for L689,560.

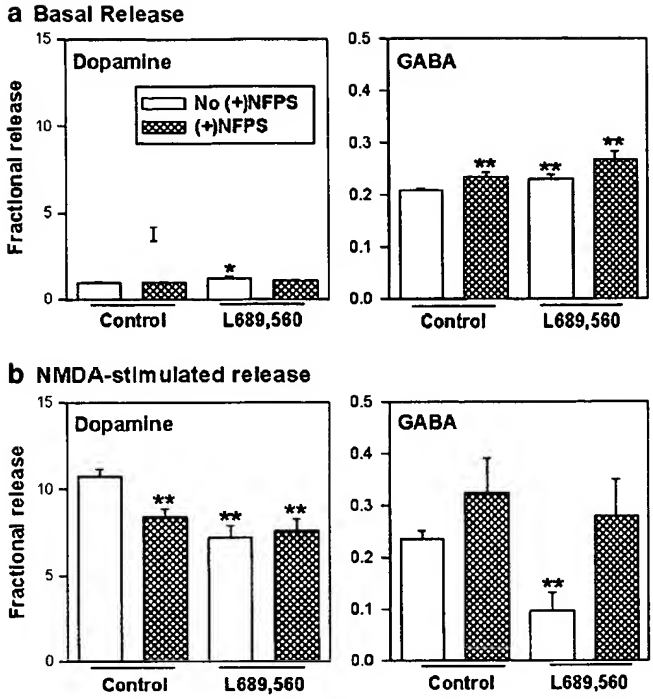

Figure 5 Effect of (+)NFPS (10 μM) on basal (a) and NMDA-stimulated (b) DA and GABA release in the absence and presence of L689,560 (100 nM). *$p<0.05$ *vs* ctl; **$p<0.01$ *vs* control.

Effect of $GABA_B$ Ligands

A final series of experiments evaluated potential mechanisms by which GTIs mediate their effects. The most likely basis for the inhibitory effects is a modulation of GABAergic neurotransmission. GABA mediates its effects at $GABA_a$ receptors, which are sensitive to the selective $GABA_a$ antagonist bicuculline, and $GABA_b$ receptors, which are sensitive to the selective agonist baclofen and the selective antagonists phaclofen and CGP52432. Bicuculline had no significant effect on [^{3}H]DA release either on its own or in combination with (+)NFPS (data not shown). Subsequent studies, therefore, examined effects of $GABA_b$ ligands.

In this experiment, a dose of (+)NFPS of 25 μM was used (Figure 6). As in previous experiments, (+)NFPS produced a highly significant reduction in fractional [^{3}H]DA release ($t=4.54$, df = 262, $p<0.001$). Experiments were repeated in the presence of the $GABA_b$ receptor agonist baclofen (100 μM), and the antagonists phaclofen (50 μM) and CGP52432 (1 μM). Baclofen on its own significantly inhibited DA release ($t=4.91$, df = 166, $p<0.001$). Moreover, in the presence of baclofen, (+)NFPS produced no further significant inhibition, and even a tendency toward significant increase ($t=1.81$, df = 35, $p=0.08$), leading to a

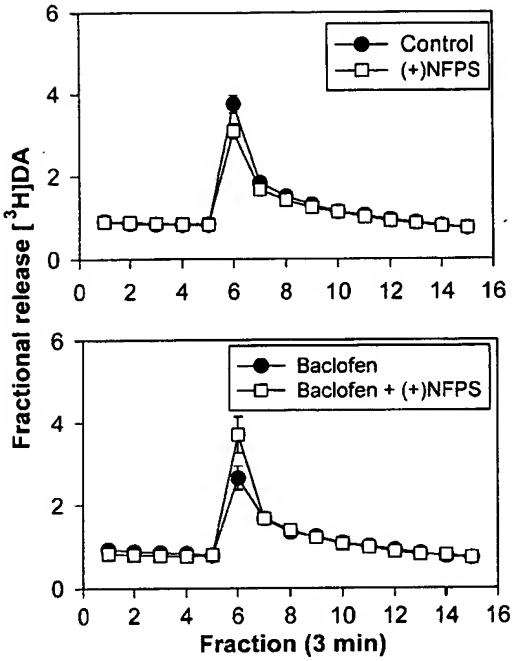

Figure 6 Effect of (+)NFPS (25 μM) on NMDA (300 μM)-stimulated [^{3}H]DA release in the absence and presence of the $GABA_B$ agonist baclofen (100 μM).

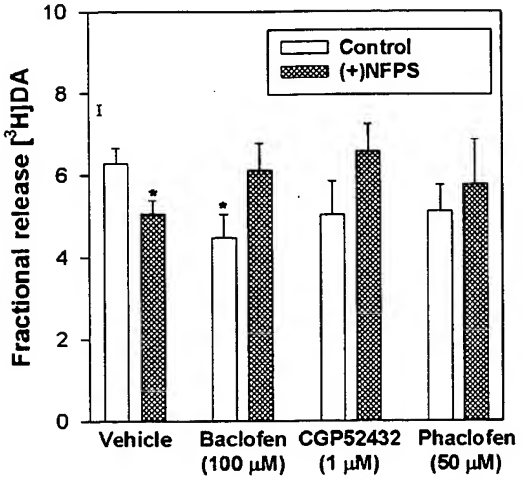

Figure 7 Effects of (+)NFPS (25 μM) on NMDA (300 μM)-stimulated [^{3}H]release in the absence and presence of the $GABA_B$ agonist bacofen (100 μM) and the $GABA_B$ antagonists CGP52432 (1 μM) and phaclofen (50 μM). *$p<0.05$ *vs* control.

significant NFPS × baclofen interaction ($F=8.7$, df = 1/164, $p=0.004$) (Figure 7).

No significant reduction in [^{3}H]DA release was observed following treatment with the $GABA_b$ antagonists phaclofen ($t=1.07$, df = 76, $p=0.3$) or CGP52432 ($t=1.41$, df = 76, $p=0.6$) (Figure 7). Nevertheless, in the presence of $GABA_b$ antagonists, (+)NFPS was without effect. In the CGP52432 experiments, there was no significant effect of either CGP52432 ($F=0.9$, df = 1/211, $p=0.3$) or (+)NFPS ($F=0.8$, df = 1/211, $p=0.4$), but the CGP52432 × (+)NFPS interaction was significant ($F=6.1$, df = 1/211, $p=0.015$), reflecting the differential (+)NFPS effect in the absence *vs* presence of $GABA_b$ antagonists. Similarly, in the phaclofen experiments, an ANOVA revealed no significant effect of either phaclofen ($F=1.7$, df = 1/211, $p=0.2$) or (+)NFPS ($F=1.8$, df = 1/211, $p=0.2$). However, the phaclofen− × (+)NFPS interaction was significant ($F=4.0$, df = 1/211, $p=0.045$), replicating the CGP52432 results.

DISCUSSION

Current treatments for schizophrenia target the dopamine system primarily by blockade of dopamine D_2 receptors. More recent theories have suggested alternative use of drugs targeted at the NMDA receptor (Javitt, 2001; Javitt and Zukin, 1991). 'First-generation' agents that have been used in clinical studies include both glycine (Heresco-Levy *et al*, 2004; Heresco-Levy *et al*, 1999; Javitt *et al*, 2001; Javitt *et al*, 1994) and D-serine (Tsai *et al*, 1998), which are endogenous agonists, as well as the synthetic glycine/D-serine derivative D-cycloserine, which is a partial agonist (Goff *et al*, 1999; Heresco-Levy *et al*, 1998). Development of high-affinity glycine-site agonists, however, is limited by the small molecular size of the target (McBain *et al*, 1989). A more recent approach has been the development of GTIs (Atkinson *et al*, 2000; Bergeron *et al*, 1998; Javitt, 2002; Javitt and Frusciante, 1997). This approach is analogous to use of selective noradrenaline or serotonin reuptake inhibitors, rather than noradrenaline or serotonin precursors, to influence brain monoamine levels in depression, or use of GABA transport inhibitors to elevate brain GABA levels in epilepsy or other brain hyperexcitability states (Dalby, 2003). The primary finding of the present study is that high-affinity GTIs show significant *in vitro* efficacy in potentiating NMDA-stimulated GABA release in striatum, and that this effect leads to significant inhibition of striatal DA release, an effect that would be expected to be therapeutically beneficial in schizophrenia.

The present results support a model in which NMDA receptors have dual excitatory–inhibitory function within striatum (Leviel *et al*, 1989; Taber *et al*, 1996). The excitatory effects of glutamate are most likely mediated at NMDA receptors located on presynaptic dopamine terminals. The existence of such receptors is well documented (Gracy and Pickel, 1996; Krebs *et al*, 1991; Wang, 1991). However, ultrastructual evidence of functional axoaxonal glutamatergic synapses on DA terminals is lacking, suggesting that the receptors may have limited physiological relevance. In the present study, NMDA stimulation of DA release via these receptors serves to mimic the impulse-dependent DA release that would normally be elicted by corticonigral activation in response to psychostimulant agents such as amphetamine, permitting analysis of feedback mechanisms within the system. Inhibitory effects of NMDA are mediated through NMDA receptors located on intrinsic GABAergic interneurons. Infusion of NMDA into striatum in intact animals increases both DA and GABA release (Hernandez *et al*, 2003), consistent with this model. Further, GABAergic inhibitory mechanisms in striatum have been demonstrated in both rodent (Hanania and Johnson, 1999; Hernandez *et al*, 2003) and primate (Schiffer *et al*, 2003) striatum, demonstrating *in vivo* relevance of this pathway to intrinsic DA regulation.

In brain, extracellular glycine levels are sufficient to fully saturate the NMDA/glycine-binding site. GTIs are postulated to function by regulating synaptic, relative to extracellular, levels, leading to local protection of the NMDA/glycine-binding sites (Supplisson and Bergman, 1997). In the present study, GTIs did not affect presynaptic DA release, presumably due to the absence of functional synapses and of a protected intrasynaptic space , but did increase NMDA-stimulated GABA release consistent with the existence of ultrastructurally demonstrated glutamatergic synapses on GABAergic interneurons. In contrast, L689,560, which inhibits glycine binding at both presynaptic and postsynaptic receptors, inhibited both DA and GABA release. GTIs were without significant effect on DA release in the presence of L689,560, confirming the role of increased glycinergic stimulation at the NMDA/glycine site in mediating their effects. Interestingly, while GTIs stimulated striatal GABA release and L689,560 inhibited release, there was no significant interaction between the two treatments. This may indicate hetereogeneity either of GABA/NMDA interactions or NMDA subtypes in striatum (Nankai *et al*, 1995). Nevertheless, both effects were in the predicted direction, with presumed NMDA stimulators increasing striatal GABA release and the NMDA antagonist decreasing GABA release.

The role of dopamine in the pathophysiology of schizophrenia has been appreciated for at least the past 40 years. Recent *in vivo* imaging studies have confirmed the relation between striatal dopamine release and have suggested that increased striatal dopamine levels may serve as a final common mechanisms for generation of positive psychotic symptoms. Thus, striatal dopamine levels are increased not only by DA agents such as amphetamine (Laruelle *et al*, 1999) but also by NMDA antagonists such as PCP and ketamine (Breier *et al*, 1998), with the degree of increase correlating with the level of psychotic symptoms. Further, normalization of striatal dopamine release accompanies clinical remission of positive symptoms in schizophrenia despite persistence of negative symptoms (Laruelle *et al*, 1999). The ability of glycine (Javitt *et al*, 2000) and GTIs (this study) to reduced striatal dopamine release is consistent with recent observations that NMDA agonists such as glycine (Heresco-Levy *et al*, 2004), D-serine (Heresco-Levy *et al*, submitted; Tsai *et al*, 1998), and the naturally occurring GTI sarcosine (Tsai *et al*, 2004) significantly ameliorate persistent positive, as well as negative, psychotic symptoms in schizophrenia, with positive symptom effects most likely reflecting effects on striatal NMDA/DA interactions.

Finally, the present study demonstrates a significant involvement of $GABA_B$ receptors in mediating the effects of NMDA-stimulated GABA release on DA inhibition. This effect is consistent with known localization of $GABA_B$ receptors to presynaptic DA terminals (Smolders *et al*, 1995), and the ability of $GABA_B$ agonists to partially inhibit NMDA-induced striatal tyrosine hydroxylase stimulation in NMDA (Arias Montano *et al*, 1992; Arias-Montano *et al*, 1991).

Although the present study focuses on GTI potentiation of NMDA receptor-mediated neurotransmission within striatum, this is unlikely to be the sole site of psychotherapeutic action of these agents. GTIs, for example, have been shown to stimulate NMDA receptor-mediated neurotransmission in both hippocampus (Bergeron *et al*, 1998) and prefrontal cortex (Chen *et al*, 2003), areas of the brain associated with prominent disability and negative symptom generation in schizophrenia. The present results, however, suggest that *even* DA dysregulation in schizophrenia may be attributable to NMDA dysfunction, and that NMDA stimulatory agents may exert significant psychotherapeutic effects even for positive symptoms. A limitation of the study is that an *in vitro* model system was used. Replication of these results *in vivo* with more recently developed, systematically active GTIs, therefore, will be critical.

In summary, this is the first study to demonstrate significant modulation of striatal dopamine release by high-affinity GTIs, and one of small number of studies to date validating the GTI concept as a method for stimulating systems-level NMDA receptor-mediated neurotransmission. These studies are encouraging of continued development of GTIs as novel psychotherapeutic agents in schizophrenia and related neuropsychiatric disorders.

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The glycine transporter-1 inhibitors NFPS and Org 24461: a pharmacological study

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Abstract

The in vitro and in vivo pharmacology of two glycine transporter-1 (GlyT1) inhibitors, *N*[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)-propyl]sarcosine (NFPS) and *R,S*-(+/−)*N*-methyl-*N*-[(4-trifluoromethyl)phenoxy]-3-phenyl-propylglycine (Org 24461), was studied. NFPS and Org 24461 inhibited the uptake of [^{3}H]glycine in hippocampal synaptosomal preparation with IC_{50} values of 0.022 and 2.5 μM. Neither NFPS nor Org 24461 (0.1 μM) showed significant binding to α-1, α-2, and β-adrenoceptors, D_1 and D_2 dopamine receptors, and 5-HT_{1A} and 5-HT_{2A} serotonin receptors in membranes prepared from rat brain or to cloned 5-HT_6 and 5-HT_7 receptors. At 10 μM concentrations, binding affinity was measured for NFPS to 5-HT_{2A} and 5-HT_{2C} serotonin receptors and α-2 adrenoceptors and for NFPS and Org 24461 to 5-HT_7 serotonin receptors. Glycine (0.1 mM) and sarcosine (5 mM) increased [^{3}H]glycine efflux from superfused rat hippocampal slices preloaded with [^{3}H]glycine. NFPS and Org 24461 (0.1 mM) did not influence [^{3}H]glycine efflux, however, they inhibited glycine-induced [^{3}H]glycine release. These findings indicate that NFPS and Org 24461 selectively inhibit glycine uptake without being substrates of the transporter protein. Several antipsychotic tests were used to characterize antipsychotic effects of NFPS and Org 24461 in vivo. These compounds did not alter apomorphine-induced climbing and stereotypy in a dose of 10 mg/kg po in mice and did not induce catalepsy in a dose of 10 mg/kg ip in rats. The ID_{50} values of NFPS were 21.4 mg/kg and higher than 30 mg/kg ip for inhibition of phencyclidine (PCP)- and D-amphetamine-induced hypermotility in mice and these values were 2.5 and 8.6 mg/kg ip for Org 24461. NFPS and Org 24461 did not exhibit anxiolytic effects in light–dark test in mice, in the *meta*-chlorophenylpiperazine (mCPP)-induced anxiety test (minimal effective dose or MED was higher than 3 mg/kg ip) and in the Vogel conflict drinking test in rats (MED was higher than 10 mg/kg ip). Both NFPS and Org 24461 (1–10 mg/kg ip) reversed PCP-induced changes in EEG power spectra in conscious rats. These data support the view that GlyT1 inhibitors may have potential importance in treatment of negative symptoms of schizophrenia.

Keywords: NFPS; Org 24461; [^{3}H]Glycine uptake; Glycine transporter-1 inhibitors; [^{3}H]Glycine efflux; Antipsychotic agents; Antipsychotic tests; Negative symptoms of schizophrenia; Anxiolytic tests; Electroencephalogram

1. Introduction

Novel antipsychotic agents that block D_2 dopamine and 5-HT_2 receptors exhibit favorable side effect profile although their clinical efficacy in blocking some symptoms of schizophrenia has certain limits (Zimbroff et al., 1997). Despite improved antipsychotic effect of clozapine, there remains a population of patients irresponsive to clozapine as its impact upon primary negative symptoms may be limited (Kane and Freeman, 1994; Meltzer, 1995). In addition, atypical antipsychotic drugs that act on 5-HT_{2A} receptors are often delayed and not fully restorative (Tamminga, 1998a). The lack of full efficacy of antipsychotics may be explained by the fact that not only dopaminergic and

Abbreviations: NFPS, *N*[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)-propyl]sarcosine; Org 24461, *R,S*-(+/−)*N*-methyl-*N*-[(4-trifluoromethyl)phenoxy]-3-phenyl-propylglycine.

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serotonergic but other neurotransmission mechanisms are also involved in the pathology in schizophrenia. Hypoactivity of *N*-methyl-D-aspartate (NMDA) receptor-mediated glutamatergic neurotransmission has been implicated in induction of negative symptoms of schizophrenia (Javitt and Zukin, 1991; Coyle, 1996). Although the mechanisms by which reduced activity of NMDA receptor-mediated glutamatergic tone occurs are not known, dysregulation of glutamate receptor expression or altered metabolism in glutamatergic transmission has been suggested (Tsai et al., 1998).

Competitive NMDA antagonists that were developed primarily to treat brain ischemia exhibit psychotomimetic effects (Kristensen et al., 1992; Grotta et al., 1995). Moreover, phencyclidine (PCP) and other noncompetitive NMDA antagonists also induce schizophrenia-like psychotic symptoms in humans as they induce hallucinations and delirium, thought disturbance and cognitive dysfunction and also negative symptoms (Heresco-Levy et al., 1996; Lahti et al., 2001). Thus, PCP, which blocks the action of glutamate at NMDA receptors, gives further support to the hypoglutamatergic theory of schizophrenia (Tamminga, 1998b). PCP has been proposed to be a pharmacological model of schizophrenia because it can mimic the full spectrum of schizophrenic disorders (Moghaddam and Adams, 1998).

An important breakthrough in understanding NMDA receptor-mediated glutamatergic neurotransmission was the recognition of glycine as a coagonist of glutamate at this ion channel-coupled receptor (Johnson and Ascher, 1987; Berger et al., 1998). Glycine and glutamate act as cotransmitters for opening NMDA-sensitive ionotropic glutamate receptors in a strychnine-insensitive manner (Foster and Kemp, 1989) influencing the permeability of the receptor-coupled ion channel for mono- and bivalent cations (Reynolds and Miller, 1988). NMDA receptors consist of NR1 and NR2 subunits, the former possesses binding site for glycine ($glycine_B$ binding site) and the latter binds glutamate to the agonist binding site (Parsons et al., 1998). Certain D-amino acids like D-cycloserine or D-serine exert partial agonist effects at $glycine_B$ site (Tsai et al., 1998; Mothet et al., 2000; Snyder and Kim, 2000) whereas kynurenic acid and some other compounds (2-carboxyindoles, 2-carboxy-tetrahydroquinolines, 4-hydroxy-2-quinolones, quinoxaline-2,3-diones) act as antagonists at the glycine coagonist site of NMDA receptors (Danysz and Parson, 1998). Activation of NMDA receptors requires occupancy of $glycine_B$ binding sites by endogenous glycine released from neighboring cells into the vicinity of NMDA receptors. A potent glycine transport mechanism assures to set glycine concentrations below the level required to saturate glycine sites at NMDA receptors (Sato et al., 1995). The high-affinity glycine transport system, which primarily inactivates glycine in glutamatergic synapses, is located in membranes of neurons and glial cells (Zafra et al., 1995).

Glycine transporters are members of the Na^+- and Cl^--dependent neurotransmitter transporter family (Uhl and Hartig, 1992; Amara et al., 1995). Two glycine transporter genes (GlyT1 and GlyT2) have been identified and cloned (Adams and Kuhar, 1993; Kim et al., 1994). In addition, three isoforms for GlyT1 (GlyT1a, GlyT1b, and GlyT1c) and two isoforms for GlyT2 (GlyT2a and GlyT2b) have been identified, cloned, and characterized (Borowsky and Hoffman, 1998; Pronce et al., 1998). Glycine transporters, which regulate glycine concentrations in excitatory synapses, belong to GlyT1 transporters and transport proteins have been shown to be colocalized with NMDA receptors (Smith et al., 1992; Fedele et al., 1993).

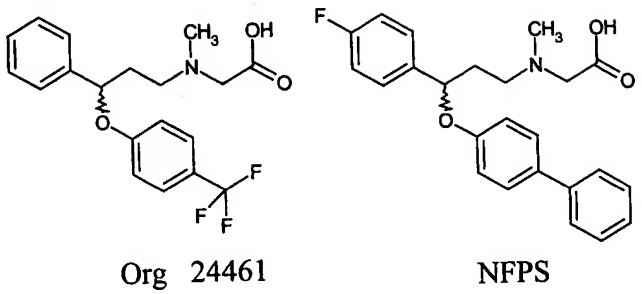

Fig. 1. The chemical structures of Org 24461 and NFPS.

A number of compounds have been reported to have selective and high affinity to glycine transporters. Toth and Lajtha (1986) and Toth et al. (1986) have demonstrated that glycine inhibits PCP-induced hyperactivity in mice and of the glycine derivatives, glycyldodecylamide (GDA) was found particularly active in this respect. Later, the glycine reuptake inhibitory effect of GDA has been demonstrated (Javitt and Frusciante, 1997; Javitt et al., 1999). In the glycine transporter inhibitor *N*[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)-propyl]sarcosine (NFPS) and *R,S*-(+/−)*N*-methyl-*N*-[(4-trifluoromethyl)phenoxy]-3-phenyl-propylglycine (Org 24461), the amino group of sarcosine was substituted with lipophilic heterocycles (Bergeron et al., 1998; Ge et al., 1999; Atkinson et al., 2000). The aim of the present investigation was to further characterize the pharmacology of the GlyT1 inhibitor Org 24461 and NFPS (Fig. 1) in in vitro and in vivo models. It was found that these GlyT1 inhibitors reverse PCP-induced alterations suggesting that they may have a therapeutic role in influencing negative symptoms of schizophrenia. A preliminary report of these findings was presented at the 35th Winter Conference of Brain Research (Harsing et al., 2002).

2. Methods

2.1. Animals

The experimental protocol used in this study was approved by the Ethic Committee, Division of Preclinical Research at EGIS Pharmaceuticals and all procedures are in compliance with the National Institute of Health Guide for

Care and Use of Laboratory Animals. Male Wistar rats weighing 260–300 g were used in the experiments. All rats were obtained from Charles River, Hungary except those tested for electroencephalogram (EEG), which were raised in the laboratory. In some experiments, male NRMI mice weighing 25–33 g were used and purchased from Charles River, Hungary. Animals were housed five to a cage in a temperature- and humidity-controlled animal facility on a 12-h light–dark cycle (6.00 a.m. on; 6.00 p.m. off) with food and water available ad libitum. The animals were allowed at least 1 week of habituation to their housing prior to experimentation.

2.2. Drugs used in this study

NFPS (ALX-5407) and Org 24461 were synthesized by Dr. P. Matyus. D-Amphetamine sulfate, apomorphine HCl, butaclamol HCl, 1-(3-chlorophenyl)piperidine (mCPP) 2HCl, clozapine, cyproheptadine HCl, diazepam, glycine HCl, haloperidol, methiotepine mesylate, mianserin HCl, MK-801, phentolamine mesylate, prazosin HCl, ritanserin, sarcosine HCl, *R*(+)SCH-23390 HCl, and serotonin creatinine sulfate were purchased from Sigma (St. Louis, MO, USA). Buspirone, PCP, and risperidone were synthesized at EGIS Pharmaceuticals, Budapest, Hungary. [^{3}H]Glycine, [^{3}H]MK-801, [^{3}H]ketanserin, and [^{3}H]LSD were purchased from New England Nuclear Life Science Products (Boston, MA, USA). [^{3}H]8-OH-DPAT, [^{3}H]mesulergine, [^{3}H]prazosin, [^{3}H]idazoxan, [^{3}H]SCH-23390, and [^{3}H]spiperone were obtained from Amersham Life Sciences, UK. All other chemicals were of analytical grade.

2.3. Neurochemical studies

2.3.1. Receptor binding assays

Competition binding studies for the GlyT1 inhibitors and some antipsychotic drugs were performed at NMDA receptors, multiple dopaminergic and serotonergic receptors, and adrenoceptors. The protocols employed for examination of drug affinities at specific receptor types are summarized in Table 1.

2.3.2. Glycine uptake in hippocampal synaptosome

Synaptosomal P_2 fraction was prepared as described by Gray and Whittaker (1962). Rats were decapitated, the brains were removed from the skull and the hippocampi were dissected and placed into ice-cold saline. The tissue was homogenized in 40 volumes (w/v) of 0.32 M sucrose. The homogenate was centrifuged at $1000 \times g$ for 5 min to remove nuclei and debris, then the supernatant was centrifuged again at $12{,}000 \times g$ for 20 min. The P_2 pellet was resuspended in Krebs solution with the following composition (in mM): NaCl 125, KCl 3, $CaCl_2$ 1.2, NaH_2PO_4 1, $MgSO_4$ 1.2, $NaHCO_3$ 22, glucose 10, pH 7.4, and gassed with 95% O_2/5% CO_2. [^{3}H]Glycine (specific activity 41.1 Ci/mmol) uptake in synaptosomal

Table 1
Conditions for competition binding at monoaminergic receptors (summarized experimental conditions)

Receptor	Rat NMDA	Rat 5-HT$_{1A}$	Rat 5-HT$_{2A}$	Pig 5-HT$_{2C}$	Human 5-HT$_6$	Human 5-HT$_7$	Rat α_1	Rat α_2	Rat D$_1$	Rat D$_2$
Tissue	forebrain	frontal cortex	frontal cortex	choroid plexus	HEK293 cells	CHO cells	frontal cortex	frontal cortex	striatum	striatum
Radioligand (nM)	[^{3}H]MK-801 (5)	[^{3}H]8-OH-DPAT (0.8)	[^{3}H]ketanserin (1)	[^{3}H]mesulergine (1)	[^{3}H]LSD (3)	[^{3}H]LSD (5.5)	[^{3}H]prazosin (0.3)	[^{3}H]idazoxan (8.9)	[^{3}H]SCH-23390 (2.8)	[^{3}H]spiperone (0.6)
Nonspecific ligand (μM)	MK-801 (10)	5-HT (10)	cyproheptadine (10)	mianserin (1)	5-HT (100)	clozapine (25)	prazosin (1)	phentolamine (10)	(+)SCH-23390 (1)	(+)butaclamol (1)
Buffer components and concentrations (mM)	HEPES (5)–NaOH glutamate (0.01), glycine (0.01)	Tris (50), $CaCl_2$ (6.66), pargyline (0.01666), ascorbic acid (0.166% w/v)	Tris (50)	Tris (50), $CaCl_2$ (4), pargyline (0.01), ascorbic acid (0.1% w/v)	Tris (50), $MgCl_2$ (10), EDTA (0.5)	Tris (50), $MgSO_4$ (10), EDTA (0.5)	Tris (50)	Tris (50)	Tris (50), $MgSO_4$ (5), EDTA (1)	Tris (50), NaCl (120), KCl (5), $CaCl_2$ (2), $MgCl_2$ (1), ascorbic acid (0.1% w/v)
pH	7.4	7.7	7.7	7.7	7.4	7.4	7.7	7.7	7.4	7.4
Incubation	120 min, 25 °C	30 min, 25 °C	15 min, 37 °C	30 min, 37 °C	60 min, 37 °C	120 min, 27 °C	45 min, 25 °C	30 min, 25 °C	30 min, 37 °C	15 min, 37 °C
Reference ligand (K_i, nM)	MK-801 (9)	buspirone (19)	ritanserin (0.9)	mianserin (2.3)	methiothepin (0.62)	methiothepin (0.14)	prazosin (0.15), WB4101 (3.8)	phentolamine (6.7), clonidine (8.6)	(+)SCH-23390 (1.8)	haloperidol (4.8)
Literature reference	Foster and Wong (1987)	Peroutka (1986)	Leysen et al. (1982)	Pazos et al. (1985)	according to information sheet	according to information sheet	Reader et al. (1987) and Greengrass and Brenner (1979)	Mallard et al. (1992) and Reader et al. (1987)	Wallace et al. (1989) and Billard et al. (1984)	Creese et al. (1979)

P_2 fraction was carried out as described by Fedele and Foster (1992). Aliquots of the P_2 fraction (0.25 mg protein) were preincubated in 2 ml of Krebs solution for 5 min at 37 °C. [^{3}H]Glycine was then added and the incubation continued for 3 min. The assay mixture was filtered under vacuum through Whatman GF/B filters that were rapidly washed three times with 5 ml of ice-cold Krebs solution. Blanks were determined by incubation at 0 °C. Filters were placed into scintillation vials with 3 ml of scintillation liquid and radioactivity was measured by liquid scintillation spectrometry. To achieve the desired ligand concentrations, [^{3}H]glycine was diluted by adding unlabeled glycine. In the experiments, 0.25 μM [^{3}H]glycine (Herdon et al., 2001) or 30 μM [^{3}H]glycine (Fedele and Foster, 1992) was used and the drugs tested were present from the beginning of the experiments at concentrations of 1–100 μM. Protein concentrations were determined by the Bradford assay using bovine serum albumin as a standard (Bradford, 1976).

2.3.3. [^{3}H]Glycine efflux from rat hippocampal slices

Rats were killed by decapitation and the brains were removed from the skulls. Slices approximately 350 μm thick from the hippocampus were prepared using a McIlwain tissue chopper (The Mickie Laboratory Engineering, Gomshall, UK). The slices were collected into ice-cold Krebs–bicarbonate buffer, pH 7.4 with the following composition (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 1.25, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $NaHCO_3$ 25, glucose 11.5. The Krebs–bicarbonate buffer used throughout the experiments was continuously gassed with 5% CO_2 in O_2. The brain slices were incubated with [^{3}H]glycine (2.5 μCi/ml) in Krebs–bicarbonate buffer for 30 min at 36 °C (Harsing et al., 2001). The tissues were then transferred into low volume (0.3 ml) superfusion chambers (Experimetria, Budapest, Hungary) and superfused with aerated and preheated (37 °C) Krebs–bicarbonate buffer. The flow rate was kept at 1 ml/min by a Gilson multichannel peristaltic pump (type M312, Villiers-Le-Bel, France). The superfusate was discarded for the first 60-min period of the experiments then 22 three-minute fractions were collected by a Gilson multichannel fraction collector (type FC-2038, Middleton, WI, USA). The GlyT1 inhibitors were added to the buffer from fraction 5 and maintained throughout the experiments. At the end of superfusion, the tissue was collected from the superfusion chambers, homogenized, and an aliquot was processed for determination of tissue content of radioactivity. To determine the radioactivity released from the tissue, a sample of the superfusate was mixed with liquid scintillation reagent (Optifluor, Packard, Groningen, The Netherlands) and subjected to liquid scintillation spectrometry. The efflux of [^{3}H]glycine was expressed as a fractional rate, i.e., as a percentage of the amount of radioactivity in the tissue at the time the release was determined (Harsing et al., 1992). A computer program (Quattro Pro) was used to estimate the fractional rate of [^{3}H]glycine efflux.

2.4. Behavioral studies

2.4.1. Apomorphine-induced climbing and stereotypy in mice

Male NMRI mice of 20–26 g were used. The test compound or the vehicle was administered orally in a volume of 20 ml/kg at the beginning of the test. Thirty minutes after the administration of the vehicle or test compound, the animals were placed individually into the experimental chamber (a wire mesh cage of 12 × 12 × 12 cm) for habituation. At the end of the 60-min pretreatment period, 1 mg/kg apomorphine HCl was administered subcutaneously to the animals. The measurement of stereotypy started immediately after the apomorphine treatment and

Table 2
Radioligand binding affinities of Org 24461 and NFPS as compared to antipsychotic drugs at multiple receptors

Receptors	Percent inhibition at different concentrations				K_i (nM)		
	Org 24461		NFPS		Haloperidol	Clozapine	Risperidone
	10^{-7} M	10^{-5} M	10^{-7} M	10^{-5} M			
NMDA	–	2	–	2	–	–	–
5-HT_{1A}	2	2	3	4	31,000	207	610
5-HT_{2A}	3	0	1	33	122	19.2	0.5
5-HT_{2C}	15	19	25	58	7900	5.8	36
5-HT_6	0	6	0	0	12,000	6	4053
5-HT_7	12	24	5	21	398	47	0.7
α-1	3	0	2	9	21	21	1
α-2	5	0	0	25	9800	230	6
β	1	0	3	9	–	–	–
D_1	4	1	2	6	131	435	148
D_2	0	1	0	3	5	630	5

Radioligand binding affinities were expressed as percent inhibition at 10^{-7} or 10^{-5} M concentrations for Org 24461 and NFPS or as K_i (inhibition constant, nM) for the antipsychotic agents. Data are means of at least two determinations performed in triplicate. For receptor binding assay conditions, see the Methods section and Table 1.

lasted 25 min. Stereotypy was scored from 0 to 4 according to the following criteria:

0 = absence of stereotyped behavior, similar to control;
1 = presence of continuous exploration, intermittent sniffing, and movements of head;
2 = intense stereotyped movements of head and/or sniffing, periodic exploration;
3 = intermittent licking, biting or gnawing with intense sniffing, or head weaving;
4 = intense licking or gnawing confined to a small area without exploration and locomotion.

Climbing behaviour was scored in an "all or none" manner 15 min after apomorphine administration for 10 min. The reaction was considered positive if the mouse clung with at least three paws on the grid wall.

Median of stereotypy scores for each group was calculated from the score maximums. The Mann–Whitney *U* test was used for statistical evaluations. ID_{50} values were calculated by linear regression analysis using percent inhibitions. Climbing frequency was calculated for each group. The result of the control group was considered as 100%. ED_{50} value was computed from the data of dose–effect relationship by the method of Litchfield and Wilcoxon (1949).

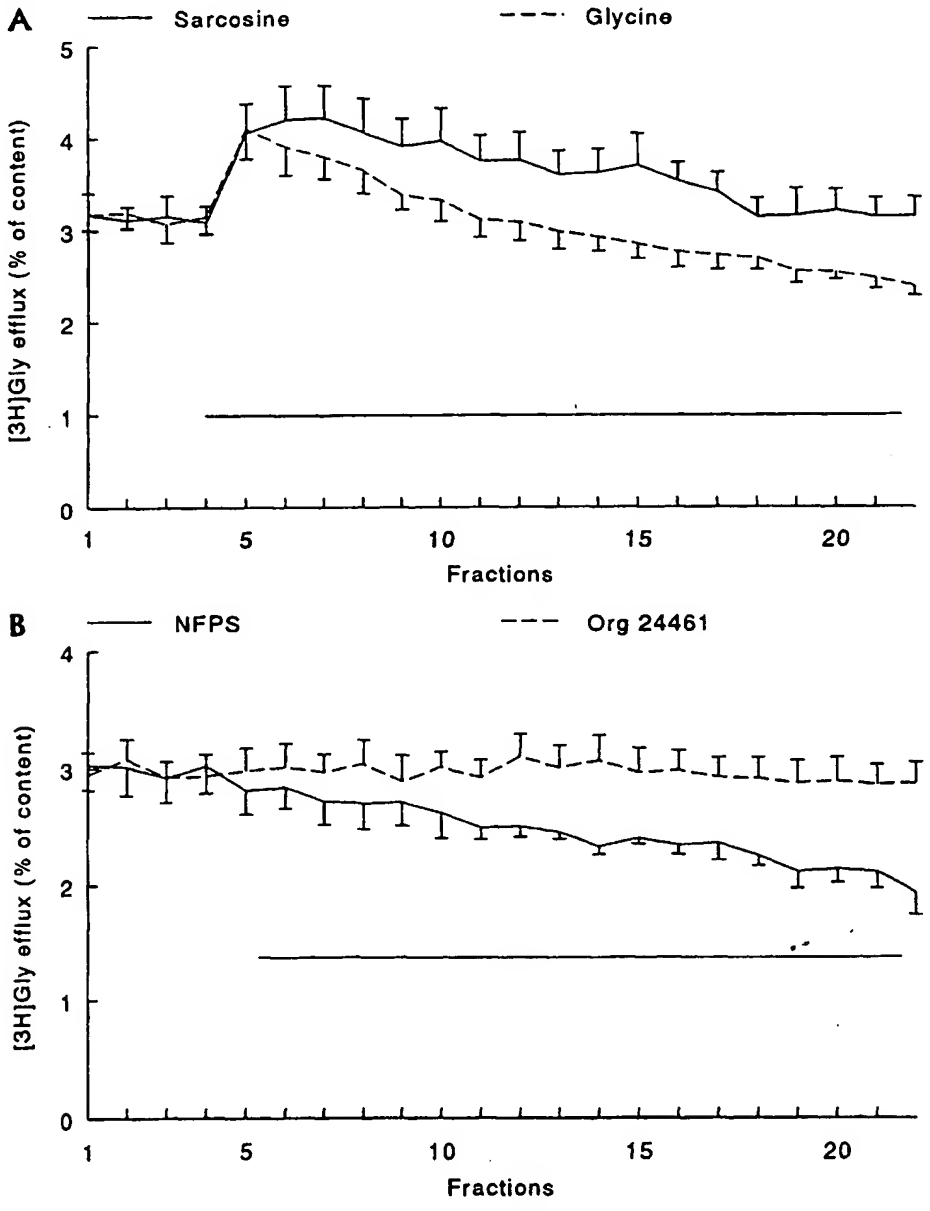

Fig. 2. Effects of GlyT1 inhibitors on [^{3}H]glycine efflux from hippocampal slices of the rat. (A) Glycine and sarcosine added in concentrations of 0.1 and 5 mM, respectively, increased [^{3}H]glycine efflux. (B) Org 24461 and NFPS added in a concentration of 0.1 mM did not influence [^{3}H]glycine efflux. Horizontal line indicates drug administration. Slices from rat hippocampus were prepared, loaded with [^{3}H]glycine, and superfused with Krebs–bicarbonate buffer. Drugs were added to hippocampal slices from fraction 5 and maintained throughout the experiments. Values shown are the means ± S.E.M. of four experiments.

2.4.2. PCP- and D-amphetamine-induced hypermotility in mice

Increasing doses of the test compound were applied intraperitoneally in a volume of 10 ml/kg to 10 mice per group. Thirty minutes later they received injections of PCP (3 mg/kg, ip). Fifteen minutes after the PCP treatment, the mice were placed in a 10-channel Dews apparatus for 45 min. The number of infrared light beam interruptions was recorded for each channel. Data were analyzed by analysis of variance (ANOVA) followed by the Duncan's test and ID_{50} values were calculated by linear regression analysis using percent inhibitions.

When effects on D-amphetamine-induced hypermotility were investigated, increasing doses of the test compound were applied intraperitoneally in a volume of 10 ml/kg to 10 mice per group. Thirty minutes later, they received injections of D-amphetamine (4 mg/kg, sc). Fifteen minutes after the D-amphetamine treatment, the mice were placed in a 10-channel Dews apparatus for 30 min. The number of infrared light beam interruptions was recorded. Data were analyzed by ANOVA followed by the Duncan's test (STATISTICA) and ID_{50} values were calculated by linear regression analysis using percent inhibitions.

2.4.3. Catalepsy in rats

The cataleptogenic effect was determined in rats by the method of Morpurgo (1962). The forepaws of rats were placed on 3- or 6-cm-high columns and an immobilization period of 10 s was scored as 0.5 or 1. A total score of 3 was considered as a 100% effect. Cataleptic activity was determined in every 30 min for a 4-h period. The Mann–Whitney *U* test was used for determination of statistical significance after the calculation of maximum score values. The compounds tested were administered intraperitoneally or orally to rats.

2.4.4. Light–dark test in mice

Test was performed in a room illuminated with a 2-lx light source. An animal activity monitor equipped with six 2-compartment automated test chambers (Omnitech, Digiscan, Model RXYZCM16) was used for all experiments. Each box consisted of one dark and one lit compartment. Both areas measured 39 × 20 × 29 cm. Access between the two compartments was provided by an 8 × 8 cm passageway. A 60-W white tungsten light bulb was used to illuminate the lit area. Interruptions of the 32 infrared beams (16 at 2 cm and 16 at 8 cm high above the box floor) in both compartments were automatically recorded by the Digiscan analyzer and transmitted to a computer. Male NMRI mice weighing 25–33 g were used for the test. Mice were kept in a dark room, treated intraperitoneally 30 min prior to test and were placed individually in the center of the lit area. Behavioral activity was detected for 5 min. Time spent in each area, number of transitions, and horizontal and vertical activities were recorded (Costall et al., 1989). Means ± S.E.M. values were calculated and the statistical analysis of data was performed by one-way ANOVA followed by the Duncan's test.

2.4.5. mCPP-induced anxiety in rats

Parameters of the experimental equipment were same as described above, except that a 40-W red tungsten light bulb was used to illuminate the lit area of the box. Male Wistar rats weighing 160–200 g were used for the test. The animals were treated intraperitoneally with vehicle or test compound. Twenty minutes later, the animals were treated subcutaneously with saline or mCPP (0.5 mg/kg). They were kept in the next 10 min in dim red light in the room where the experiment was carried out. The rats were then placed individually at the center of the dark compartment and behavioral activity was detected for 5 min. Time spent in each area, number of transitions, and horizontal and vertical activity were recorded (Bilkei-Gorzo et al., 1998). Means ± S.E.M. values were calculated and the statistical analysis of data was performed by one-way ANOVA followed by the Duncan's test.

2.4.6. Conflict drinking test in rats

Experiments were performed in a computer-operated LIIKOSYS (Experimetria) consisting of eight test chambers (20 × 20 × 20-cm Plexiglas boxes), each of which was equipped with a water fountain system mounted at an appropriate height on the wall of the chamber and metal grid floor for delivering electric shocks. Male Wistar rats weighing 160–180 g were deprived of drinking water for 48 h and fasted for 24 h prior to test. Test and reference compounds or vehicle were administered intraperitoneally 30 min prior to test. All procedures were carried out in a quiet, air-conditioned room between 0730 and 1300 h at an ambient temperature of 232 °C. At the beginning of the experiment, the animals were placed in the test chamber where they had free access to drinking water for a 30-s grace period. After that, electric shocks (600 μA, 0.6 s) were applied through the drinking spout following every 20 licks during a 5-min test period (Vogel et al., 1971). Number of

Table 3
Lack of effects of Org 24461 and NFPS on apomorphine-induced climbing and stereotypy in mice and on catalepsy in rats

Compounds	Apomorphine-induced		Catalepsy
	Climbing	Stereotypy	MED (mg/kg)
	ED_{50} (mg/kg)	ID_{50} (mg/kg)	
Org 24461	>10	>10	>10
NFPS	>10	>10	>10
Haloperidol	0.3	0.25	1
Clozapine	11	35	>40
Risperidone	0.02	0.08	1

Apomorphine-induced climbing and stereotypy were determined in mice after oral administration of the drugs as described in the Methods section. Catalepsy was determined in rats according to Morpurgo (1962). GlyT1 inhibitors were administered intraperitoneally; the antipsychotic agents were given orally. For details, see Methods section.

punished licks was recorded and stored by a computer. Means ± S.E.M. of numbers of tolerated shocks were calculated in each group, statistical analysis of data was performed by one-way ANOVA followed by the Duncan's test.

2.5. EEG testing

Male Wistar rats weighing 500 g were anesthetized by chloral hydrate (350 mg/kg ip) and mounted in a stereotaxic frame (Sebban et al., 1999). Two holes were drilled in the left and right prefrontal regions and two others in the right and left sensorimotor cortex regions, and four transcortical bipolar electrodes were inserted. The animals were earthed via a stainless steel screw fixed in the frontal cranial bone. Ten days after the surgery the animals were habituated for EEG recording for an additional 10–14 days. Two EEG recordings were performed for each dose of a drug tested in the same rat. The first recording lasted

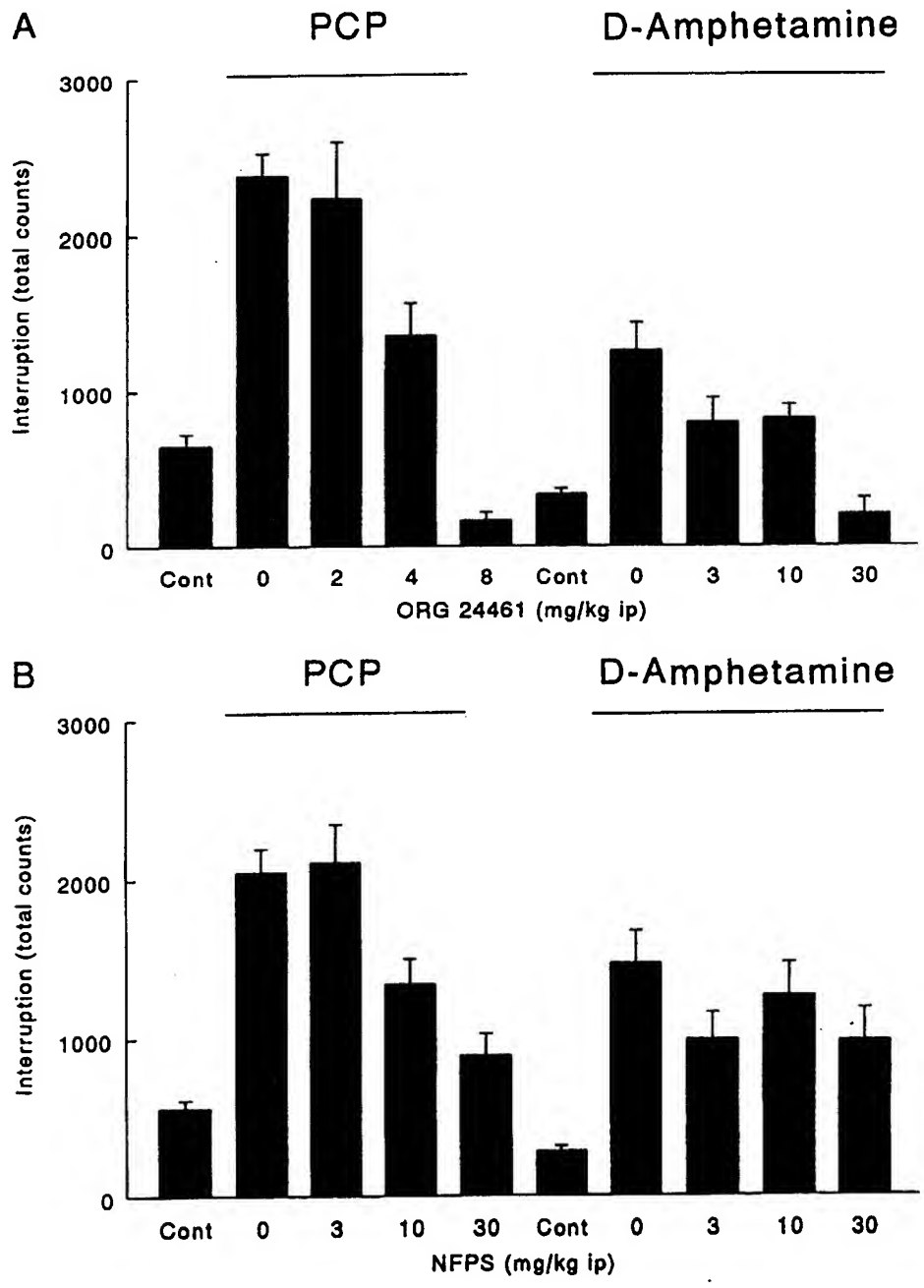

Fig. 3. Effect of Org 24461 (A) and NFPS (B) on PCP-induced and D-amphetamine-evoked hypermotility in mice. The ID_{50} values to inhibit PCP- and D-amphetamine-induced hypermotility were 3.8 and 13.5 mg/kg ip for Org 24461 and these values for NFPS were 21.4 and higher than 30 mg/kg ip. Hyperlocomotion was evoked by PCP (3 mg/kg ip) or D-amphetamine (4 mg/kg sc) as described in the Methods section. The total counts indicate the number of beams that have been interrupted by the animal during the ambulatory activity. Values shown are the means ± S.E.M. ($n = 10$).

for 165 min after vehicle administration. The second recording was done 24 h later for the same duration after drug administration. The two records were used to evaluate the treatment effect relative to the vehicle effect. For recording, EEG signals were amplified and filtered for Fourier transformation, which allowed calculation of the power variable (μV^2). Absolute power spectra of EEG signals were computed every 30 s from 1 to 30 Hz in steps of 1 Hz. In each rat, hertz by hertz drug-induced power changes were evaluated by determining spectral power obtained following the injection of PCP and drug tested versus the spectral power obtained following administration of PCP (1 mg/kg sc). The EEG spectral power from the prefrontal and sensorimotor cortices of the two hemispheres was averaged for every 5-min period for each 165-min recording session.

2.6. Statistical analyses

One-way ANOVA followed by the Dunnett's or Duncan's multiple comparison test, the Student *t* statistics for two means, and the paired *t* test were used for statistical analysis of the data. The Mann–Whitney *U* test was used for nonparametric analysis. The mean ± S.E.M. was calculated and the number of independent determinations (n) is indicated. A level of probability (P) less than 5% was considered significant.

3. Results

3.1. Neurochemical testing

3.1.1. Receptor binding assays

Using receptor binding assays, we determined the binding properties of the Org 24461 and NFPS in comparison with other antipsychotic agents. At 0.1 μM concentration, NFPS and Org 24461 did not show significant binding to α-1, α-2, and β-adrenoceptors, D_1 and D_2 dopamine receptors, and 5-HT_{1A} and 5-HT_{2A} serotonin receptors in membranes prepared from rat brain or in membranes from cells expressing recombinant h5-HT_6 and h5-HT_7 receptors (Table 2). At higher concentrations (10 μM), binding affinity was measured for NFPS at 5-HT_{2A} serotonin receptors and α-2 adrenoceptors and for NFPS and Org 24461 at 5-HT_7 serotonin receptors were measured; no receptor affinity was measured to NMDAs receptors. NFPS also showed inhibition of 5-HT_{2C} serotonin receptor binding at 0.1 and 10 μM concentrations in membranes prepared from choroid plexus, this effect of Org 24461 was less pronounced (Table 2). For comparison, the receptor binding profiles of haloperidol, clozapine, and risperidone are also shown in Table 2. These antipsychotic agents showed major affinity to a number of receptors tested. Thus, haloperidol showed affinity to α-1 and D_2 receptors, clozapine exhibited strong affinity to α-1, 5-HT_{2A}, 5-HT_{2C}, and 5-HT_6 receptors, and risperidone showed preferential affinity to 5-HT_{2A}, and α-1 and α-2 adrenoceptors and also to D_2 dopamine receptors (Table 2).

3.1.2. Effects on [^{3}H]glycine uptake in hippocampal synaptosome preparation

The uptake of [^{3}H]glycine in rat hippocampal P_2 synaptosomal preparation was found to be linear for at least 4 min. Exposition of synaptosomal P_2 fraction to hyposomotic shock led to a complete inhibition of [^{3}H]glycine uptake. The uptake of [^{3}H]glycine was Na^+-dependent as reduction of Na^+ from 148 to 23 mM in the buffer resulted in a 75% reduction of [^{3}H]glycine accumulation. Competition studies with increasing concentrations of unlabeled glycine (40–10,000 μM) revealed the presence of a high- and low-affinity glycine uptake in hippocampal synaptosomes. The high- and low-affinity glycine uptake were characterized with K_m values of 0.06 and 3 mM and maximal velocity (V_{max}) of 1.1 and 6.3 nmol/mg protein/min, respectively. These data correspond to those published in the literature (Fedele and Foster, 1992; Herdon et al., 2001). Using 0.25 μM [^{3}H]glycine concentration in the assay, we found that NFPS and Org 24461 inhibited high-affinity [^{3}H]glycine uptake with IC_{50} values of 0.022 and 2.5 μM in rat hippocampal P_2 synaptosomal preparations. These values were 2 and 28 μM for NFPS and Org 24461, respectively, when 30 μM [^{3}H]glycine was used for the determination of [^{3}H]glycine uptake.

3.1.3. Effects on [^{3}H]glycine efflux in hippocampal slices

After a 60-min preperfusion period, the spontaneous [^{3}H]glycine outflow from hippocampal slices occurred at a rate of 3.08 ± 0.15 kBq/g in 3 min (n = 4) and it decreased to 2.67 ± 0.22 kBq/g in 3 min during a 66-min period of superfusion. The content of radioactivity in hippocampal tissue was found to be 294 ± 10 kBq/g (n = 4). Efflux rate of [^{3}H]glycine from superfused hippocampal slices was increased by the addition of glycine (0.1 mM) or sarcosine

Table 4
Lack of anxiolytic effect of Org 24461 and NFPS in anxiolytic tests

Compounds	MED (mg/kg ip)		
	Light–dark test	mCPP light–dark test	Conflict drinking test
Org 24461	>3	>3	>10
NFPS	>3	>3	>10
Diazepam	0.1	0.1	5

Testing of drugs in light–dark test was carried out in mice as described by Costall et al. (1989) after intraperitoneal administration of Org 24461, NFPS, and diazepam. For determination of mCPP-induced anxiety (Bilkei-Gorzo et al., 1998), rats were treated with the test compounds intraperitoneally and 20 min later 0.5 mg/kg mCPP was injected subcutaneously. A modification of the Vogel et al.'s (1971) methods was used for the conflict drinking test in rats, Org 24461, NFPS, and diazepam were administered intraperitoneally. For details, see the Methods section.

(5 mM) to the superfusion buffer (Fig. 2). The effects of 0.1 mM Org 24461 or NFPS on [^{3}H]glycine outflow were determined in hippocampal slices in concentrations 3.5 and 50 times higher than those in inhibited [^{3}H]glycine uptake by 50%. Addition of 0.1 mM of Org 24461 or NFPS to the superfusion buffer did not affect [^{3}H]glycine outflow (Fig. 2). In addition, Org 24461 as well as NFPS (0.1 mM) inhibited the stimulatory effect of 0.1 mM glycine on

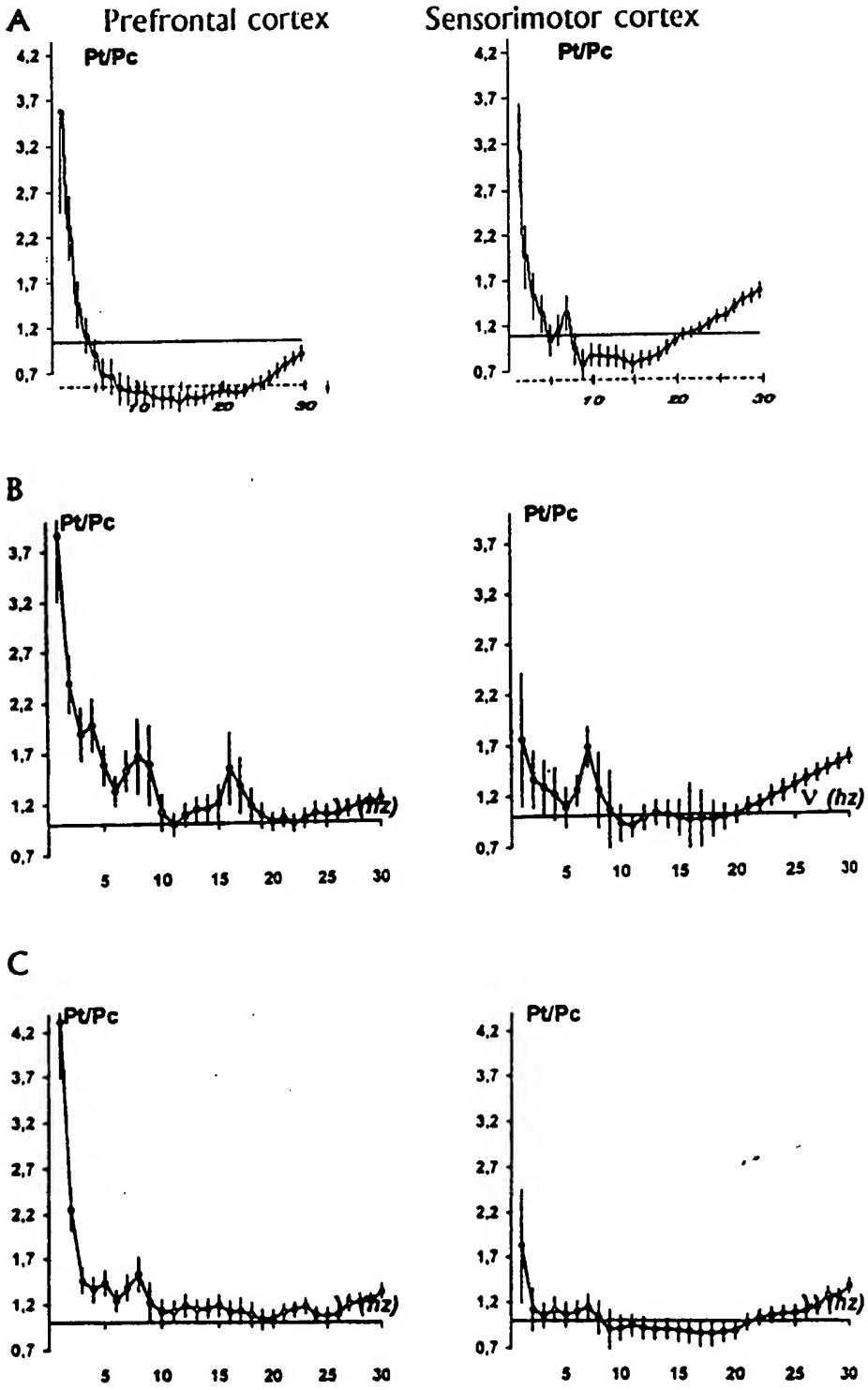

Fig. 4. Effect of Org 24461 and NFPS on PCP-induced EEG power spectral changes in prefrontal cortex (on the left) and sensorimotor cortex (on the right) of conscious rats. (A) Administration of PCP alone (1 mg/kg sc). Coadministration of PCP with Org 24461 (B) and with NFPS (C). The GlyT1 antagonists were administered in a dose of 10 mg/kg ip 30 min after PCP injection. Abscissa represents the EEG spectral component at each frequency between 1 and 30 Hz, the ordinate shows changes in the EEG power spectrum produced by drug administration. Vertical bars indicate 95% confidence intervals ($n=6$).

[^{3}H]glycine efflux. The glycine-induced [^{3}H]glycine efflux was 3.89±0.49% in the absence, and 1.67±0.16% and 2.08±0.21% of content in the presence of Org 24461 and NFPS, respectively [$F(2,9)=13.499$, $P<.01$, $n=4$].

3.2. Behavioral testing

3.2.1. Apomorphine-induced stereotypy and climbing in mice

Org 24461 and NFPS had no effect on the induction of stereotypy or climbing behavior in mice by the dopaminergic agonist apomorphine even when they were administered in an oral dose as high as 10 mg/kg (Table 3). Haloperidol and risperidone effectively inhibited apomorphine-induced stereotypy and climbing, whereas clozapine exerted only weak antagonistic effects (Table 3).

3.2.2. PCP- and D-amphetamine-induced hypermotility in mice

Org 24461 antagonized the action of PCP on locomotion with a greater potency than that of D-amphetamine; the calculated ID_{50} values were 3.8 and 13.5 mg/kg ip against PCP and D-amphetamine (Fig. 3). NFPS was less effective than Org 24461 on PCP-induced hypermotility (ID_{50} 21.4 mg/kg ip) and it was ineffective on D-amphetamine-induced hyperlocomotion in mice (ID_{50}>30 mg/kg ip, Fig. 3). The locomotion elicited by D-amphetamine was blocked by haloperidol with a far greater potency than that required to inhibit the PCP-evoked hypermotility (ID_{50} 0.05 and 1.2 mg/kg sc). The hyperlocomotion elicited by PCP or D-amphetamine was blocked by risperidone with equal potency as the ID_{50} values were 0.07 mg/kg sc in both tests. Clozapine displayed markedly greater potency against PCP- than D-amphetamine-induced hypermotility, the ID_{50} values were 2.9 and higher than 10 mg/kg sc in mice, respectively.

3.2.3. Effect of Org 24461 and NFPS on catalepsy compared with antipsychotic agents

Org 24461 and NFPS were found inactive in inducing catalepsy in rats in doses as high as 10 mg/kg (Table 3). Clozapine was also found ineffective in inducing catalepsy whereas haloperidol and risperidone elicited catalepsy in rats (Table 3).

3.2.4. Anxiolytic tests

As shown in Table 4, Org 24461 and NFPS did not induce major anxiolytic effects in light–dark test in mice or in mCPP-induced anxiety test in rats (minimal effective dose or MED was higher than 3 mg/kg ip). In conflict drinking test, Org 24461 and NFPS were also ineffective at doses of 10 mg/kg ip. Diazepam exerted anxiolytic effects in all three anxiolytic tests employed. The MED of diazepam was 0.1 mg/kg ip in light–dark test and mCPP-induced anxiety test and it was 5 mg/kg in the conflict drinking test (Table 4).

3.3. EEG power spectral analysis in prefrontal and sensorimotor cortex of conscious rats

PCP when injected in a dose of 1 mg/kg sc induced complex EEG changes according to the frequency and cortical brain region (Fig. 4). The most characteristic changes in EEG power were an increase at very low frequencies (<4 Hz) in the prefrontal and sensorimotor cortices and a power decrement at higher frequencies. EEG changes in sensorimotor cortex were mainly characterized by an increase in 7- to 8-Hz frequency band and by a further increase in power for the 20- to 30-Hz band.

Org 24461 and NFPS were injected in doses of 1, 3, and 10 mg/kg ip in order to study their effects on PCP-induced changes in EEG power spectra in conscious rats. Org 24461 and NFPS dose-dependently attenuated the power decrement evoked by PCP at higher frequencies in the prefrontal and sensorimotor cortices (Fig. 4). In addition, Org 24461, in interaction with PCP, also induced synchronization peaks at 3–5 and 8–20 Hz frequency bands in the prefrontal cortex. NFPS nearly completely inhibited the decrease in power between 5 and 30 Hz evoked by PCP and only a small synchronization at 7 Hz was observed in the prefrontal cortex (Fig. 4).

4. Discussion

The sarcosine derivative NFPS and Org 24461 inhibited [^{3}H]glycine uptake in hippocampal synaptosomal preparations and this inhibition depended on the concentration of [^{3}H]glycine used in the assay. NFPS was found to be equipotent in inhibition of glycine uptake in synaptosomes prepared from hippocampus (this study) or cerebral cortex (Herdon et al., 2001). Org 24461 inhibited glycine uptake in CHO cells expressing hGlyT1b with an IC_{50} value of 0.3 μM (Brown et al., 2001), a potency one magnitude higher than what we found in hippocampal synaptosomes. Although the reason of this discrepancy is not clear, differences in inhibition of glycine uptake determined in cell line expressing GlyT1 and synaptosomes were also demonstrated by Herdon et al. (2001). It has been reported that glycine transporter proteins, which regulate glycine concentrations in glutamatergic excitatory synapses, belong to GlyT1 transporters (Smith et al., 1992; Fedele et al., 1993). NFPS and Org 24461 inhibited glycine uptake in HEK292 cells and CHO cells expressing GlyT1c and hGlyT1b, respectively (Herdon et al., 2001; Brown et al., 2001). We therefore believe that NFPS and Org 24461 preferably inhibited GlyT1 transporter in our experimental models using hippocampal synaptosomes or slice preparations.

Brown et al. (2001) reported that Org 24598, the *R*-(−) isomer of Org 24461, showed no appreciable affinity at dopamine, serotonin, and noradrenaline receptors in radioligand binding experiments. We may confirm that the glycine uptake inhibitory effects of Org 24461 and NFPS

were associated with no major affinity to a series of neurotransmitter receptors although some receptor binding affinity of these compounds was observed at 5-HT_{2A}, 5-HT_{2C}, and 5-HT_7 serotonin receptors and α-2 adrenoceptors at the higher concentrations (10 μM) tested. Clozapine and risperidone, on the other hand, interact with multiple dopaminergic, serotonergic, and adrenergic receptors (Gunasekara et al., 2002). The interactions of these two antipsychotic agents with neurotransmitter receptor systems markedly differ from that of haloperidol, which preferentially binds to dopamine D_2 receptors. These distinctive receptor binding profiles may explain a number of differences observed in the neuropharmacological actions of GlyT1 inhibitors and antipsychotic agents tested.

To further characterize the inhibition by Org 24461 and NFPS of GlyT1 proteins, their effects were also determined on [^{3}H]glycine efflux in hippocampal slice preparations. We found that neither Org 24461 nor NFPS influenced the efflux rate of [^{3}H]glycine albeit the two GlyT1 inhibitors were added to superfused hippocampal slices in concentrations several times higher than those in inhibited [^{3}H]glycine uptake. In contrast, sarcosine and even glycine enhanced [^{3}H]glycine efflux rate in hippocampal slices. This latter finding may correlate with the observation of Herdon et al. (2001) who showed that addition of sarcosine or glycine to HEK293 cells containing hGlyT1c transporters and preloaded with [^{3}H]glycine resulted in a decrease in content of radioactivity. The fact that sarcosine and glycine enhanced, whereas Org 24461 and NFPS did not influence, [^{3}H]glycine efflux from hippocampal slices indicates different mechanisms of these compounds in glycine transporter inhibition (Aubrey and Vanderberg, 2001).

The glycine- or sarcosine-induced [^{3}H]glycine release may be consequent to the transport of these molecules into the glycine-containing cells through the glycine carriers. Glycine-releasing cells probably express homocarriers for the recapture of the released glycine and homo- or heterocarriers through which sarcosine can penetrate and evoke glycine release. The effect of sarcosine or glycine is presumably due to exchange with preloaded [^{3}H]glycine via normal operation of the transporter resulting in an increase in [^{3}H]glycine efflux. On the other hand, Org 24461 and NFPS may be true inhibitors of GlyT1 and their inhibitory effect resembles that of GDA reported earlier (Harsing et al., 2001). It is worthwhile to point out that Org 24461 and NFPS, which were without effects on [^{3}H]glycine outflow by themselves, were able to reduce the glycine-induced [^{3}H]glycine efflux from hippocampal slices. These results may suggest that both Org 24461 and NFPS are nontransportable inhibitors of GlyT1 proteins whereas sarcosine may inhibit the uptake of glycine by competing with glycine at the binding site of its transporters.

Hippocampal tissue was chosen to study the effects of GlyT1 inhibitors on glycine uptake and release as the density of $glycine_B$ binding sites associated with NMDA receptors is greatest in this brain area (Pullan and Powell, 1992). Hippocampus is also an area that has been proposed to be the primary locus of action for the dissociative action of PCP (Corssen and Domino, 1966; Miyasaka and Domino, 1968), a noncompetitive antagonist of NMDA receptors (Anis et al., 1983). In experimental animals, PCP induces complex behavior syndrome and the ability of a compound to antagonize these behavioral alterations would be predictive for its antipsychotic properties in humans (Bujas-Bobanovic et al., 2000). PCP mimics symptoms of schizophrenia by at least two mechanisms. First, PCP induces increased glutamate release in intracortical circuitry by increasing impulse flow in thalamocortical inputs (Aghajanian and Marek, 2000). Second, PCP reduces NMDA receptor-mediated functions by blocking the receptor, which then leads to disinhibition of the thalamic filter (Carlsson, 1988). Both mechanisms will result in an overload of information for the cerebral cortex leading to disruption of integrative cortical functions.

Accordingly, PCP evokes dose-dependent characteristic changes in EEG power spectra both in the prefrontal and sensorimotor cortices of conscious rats (Sebban et al., 2001). Thus, PCP increased EEG power at low frequency (1 Hz), an effect proved to be resistant to the influence of a number of drugs. Over a frequency range of 5–20 Hz, PCP decreased EEG power and the desynchronization observed at this power band may be associated with hypervigilance, which is believed to be part of schizophrenia. It was also shown that clozapine and haloperidol inhibited the PCP-induced decrease in EEG power between 5 and 30 Hz in the prefrontal cortex although differences between the actions of the two antipsychotic agents were also observed (Millan et al., 1998; Sebban et al., 2001). Thus, both clozapine and haloperidol added with PCP resulted in a synchronization at 7 Hz in the sensorimotor cortex and haloperidol evoked similar effect in the prefrontal cortex.

The GlyT1 inhibitors markedly influenced the effects of PCP on EEG spectra in the cerebral cortex and their effects resemble those of antipsychotics. NFPS homogenously attenuated the effect of PCP and these changes were characteristic for both prefrontal and sensorimotor cortices. The effect of Org 24461 was found to be more complex as this compound induced synchronization with the appearance of peaks in low (3–5 Hz) and higher (5–20 Hz) frequency bands in the prefrontal cortex. These findings suggest that NMDA receptors associated with $glycine_B$ binding sites may also be involved in the cortical desynchronization of EEG power at higher frequencies. As the PCP-induced EEG desynchronization observed at higher frequencies is likely related to an increase in cortical signal processing that corresponds to hallucination, GlyT1 inhibitors that alter this frequency range may also attenuate hallucination in schizophrenia.

The behavioral changes induced by PCP in animals consist of stereotyped behavior, ataxia, and also hyperlocomotion (Bujas-Bobanovic et al., 2000). PCP-evoked hyperlocomotion may involve serotonergic as well as dop-

aminergic mechanisms and activation of 5-HT_{2A} receptors (Martin et al., 1997; Millan et al., 1998) or increase in dopamine release in the limbic or extrapyramidal systems after PCP administration have been demonstrated (Phillips et al., 2001; Balla et al., 2001). Clozapine, an atypical antipsychotic drug with multireceptor action (Lieberman., 1993), has been reported to block hyperlocomotion induced by acute PCP administration (Maurel-Remy et al., 1995) and this effect is believed to be mediated by the blockade of 5-HT_{2A} receptors in the nucleus accumbens (Millan et al., 1998). The classic antipsychotic agent haloperidol also antagonizes increases in locomotor activity after PCP administration and this effect of haloperidol is probably due to its blockade of D_2 dopamine receptors (Kitaichi et al., 1994; Maurel-Remy et al., 1995). Another antipsychotic agent, risperidone, which shows a pharmacology that partially overlaps both haloperidol and clozapine, also blocks the development of PCP-induced locomotor hyperactivity probably by acting on D_2 and 5-HT_{2A} receptors (Kitaichi et al., 1994). Similar to the antipsychotic drugs, the GlyT1 inhibitor Org 24461 and NFPS also inhibited PCP-induced hyperlocomotion in our experiments. The fact, however, that neither Org 24461 nor NFPS expressed affinity to dopamine or 5-HT receptors in binding assays suggests that GlyT1 inhibitors influence PCP-induced locomotion by other mechanisms, probably by increasing glycine concentrations at $glycine_B$ binding sites on NMDA receptors. It may be important to call attention to the preferential effects of GlyT1 inhibitors on PCP- versus D-amphetamine-induced hyperlocomotion (see below) that may suggest their potency to control negative symptoms.

Whereas PCP elicits negative symptoms or both negative and positive symptoms of schizophrenia (Cosgrove and Newell, 1991; Javitt and Zukin, 1991), the use of direct and indirect dopamine agonists has been proposed to be the principal model for positive symptoms of schizophrenia (Arnt and Skarsfeldt, 1998). Therefore, in the first series of experiments, the indirect dopamine agonist D-amphetamine was employed to test the effects of GlyT1 inhibitors on dopamine receptor-mediated effects. D-Amphetamine induces locomotion, an effect mediated mainly by D_2 dopamine receptor stimulation in the nucleus accumbens (Arnt, 1995). We found that Org 24461 inhibited the D-amphetamine-induced hypermotility whereas NFPS was found inactive in this test. This finding suggests there might be some differences in the mechanism of action of the two GlyT1 inhibitors as Org 24461, which contains fluoxetine moiety, that may alter some other neurotransmitter system(s) at receptor or transporter levels.

In another series of experiments, we have determined the effects of Org 24461 and NFPS on apomorphine-induced climbing and stereotypy in mice. Inhibition of apomorphine-induced climbing is an effect related to D_2 dopamine receptor stimulation mainly in the limbic system and that may be predictive for antipsychotic activity of a drug (Kahn and Davis, 1995). On the contrary, inhibition of apomorphine-induced stereotypy is an effect related to D_2 dopamine receptor stimulation particularly in the extrapyramidal system, and positive data obtained in this test may predict potential extrapyramidal side effects of an antipsychotic drug candidate (Costall and Naylor, 1977; Ninan and Kulkarni, 1999). The fact that the GlyT1 inhibitor Org 24461 and NFPS did not influence apomorphine-induced climbing and stereotypy indicates that these compounds may have limited potential in mediation of limbic and extrapyramidal dopaminergic neurotransmission. Clozapine, haloperidol, and risperidone used as reference compounds in this study, however, inhibited the apomorphine-induced climbing and stereotyped behavior according to their binding potency to dopamine receptors.

Antipsychotic agents elicit an extrapyramidal motor syndrome by the interruption of activity at striatal D_2 or D_1 dopamine receptors and catalepsy induced in rats may be predictive for an extrapyramidal side effect in humans (Hoffman and Donovan, 1995). Haloperidol potently elicited catalepsy in rats, a response reflecting inhibition of activity at D_2 dopamine receptors in the striatum. Risperidone, which shows D_2 and 5-HT_{2A} affinities but no agonist effect on 5-HT_{1A} receptors, also induced catalepsy in rats further confirming the extrapyramidal side effect-inducing ability of this compound observed in humans (Gunasekara et al., 2002). Clozapine, however, was found to be less potent in inducing catalepsy and its reduced extrapyramidal side effects may be explained by marked affinity to muscarinic receptors (Brunello et al., 1995). Similarly to the apomorphine-induced stereotypy, the GlyT1 inhibitor Org 24461 and NFPS did not induce catalepsy in rats presumably suggesting the lack of extrapyramidal side effects of these compounds in patients. The lack of effect of GlyT1 inhibitors on D_2 receptors speaks for improving negative symptoms of schizophrenia with a benign extrapyramidal potential without eliciting tardive dyskinesia after a long-term treatment.

Anxious symptoms are frequently encountered in many psychiatric disorders and it was proposed that anxiolytic properties would be useful for an antipsychotic agent (Millan et al., 1999). In contrast to haloperidol, clozapine possesses some anxiolytic properties in certain experimental models (Wiley et al., 1993). Since GlyT1 inhibitors also shared antipsychotic activity in some but not all tests employed, we determined whether these compounds possess anxiolytic activity. The GlyT1 inhibitor Org 24461 and NFPS, which indirectly activate $glycine_B$ binding site by increasing glycine levels at the vicinity of NMDA receptors, failed to demonstrate anxiolytic effects in a number of tests suitable to detect anxiolytic activity. It is interesting to mention that glycine, a full agonist at $glycine_B$ binding site, was also inactive in animal models of anxiety (Winslow et al., 1990; Chojnacka-Wojcik et al., 1996). On the other hand, D-cycloserine, a partial agonist at $glycine_B$ site, showed an anxiolytic-like activity in the Vogel conflict drinking test in rats (Klodzinska and Chojnacka-Wojcik,

2000) and a similar effect has also been reported in the elevated plus maze paradigm (Karcz-Kubicha et al., 1997). In the Vogel conflict drinking test, however, the anxiolytic effect of D-cycloserine and that of ACPC, another partial agonist at glycine$_B$ binding site, was not affected by pretreatment with glycine (Chojnacka-Wojcik et al., 1996; Klodzinska and Chojnacka-Wojcik, 2000). These findings, in addition to our results obtained with GlyT1 inhibitors, may indicate that glycine$_B$ binding site at NMDA receptors is probably not directly involved in anxiogenic/anxiolytic mechanisms in the central nervous system (Klodzinska and Chojnacka-Wojcik, 2000).

In summary, the GlyT1 inhibitors Org 24461 and NFPS display marked differences in mode of action to haloperidol and the atypical antipsychotic clozapine and risperidone. Their receptor, glycine transporter inhibitory, and functional profiles suggest that they may be able to control negative symptoms of schizophrenia in the relative absence of effects on positive symptoms and extrapyramidal motor functions. Thus, GlyT1 inhibitors are likely to be of use in the treatment of schizophrenia with a narrower margin of influencing symptoms in comparison of currently used antipsychotic agents. In schizophrenia, the reduced thalamic filter may result in a higher glutamatergic tone in the cerebral cortex, which is associated with hypofunctional NMDA receptors located on postsynaptic neural substrates. GlyT1 blockers may reverse NMDA receptor hypofunction by the blockade of glycine reuptake, which then leads to increase of glycine levels in the vicinity of glycine$_B$ binding sites at NMDA receptors. The hypothesis that schizophrenia may be associated with decreased central nervous system glycine levels was further shown by the observation that high oral doses of glycine improved negative symptoms of schizophrenic subjects (Heresco-Levy et al., 1996). Thus, GlyT1 inhibitors may influence beneficially symptoms of schizophrenia by the inhibition of glycine uptake process.

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